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PATENT COOPERATION TREATY

1648

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

WATERMARK PATENT & TRADEMARK ATTORNEYS
2nd floor
290 Burwood Road
Hawthorn, VIC 3122
AUSTRALIE

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
08 August 2000 (08.08.00)	
Applicant's or agent's file reference	
International application No. PCT/AU98/00648	**International filing date** (day/month/year) 14 August 1998 (14.08.98)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

PIG RESEARCH DEVELOPMENT CORPORATION COMPUTER ASSOCIATE HOUSE
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State of Nationality	State of Residence
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2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [ ] the address [ ] the nationality [ ] the residence

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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer H. Zhou
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994) 003453052

PATENT COOPERATION TREA[illegible]

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 26 March 1999 (26.03.99)	
International application No. PCT/AU98/00648	**Applicant's or agent's file reference**
International filing date (day/month/year) 14 August 1998 (14.08.98)	**Priority date** (day/month/year) 14 August 1997 (14.08.97)
Applicant JOHNSON, Michael, Anthony et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

10 March 1999 (10.03.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Lazar Joseph Panakal
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Form PCT/IB/331 (July 1992)

2543131

14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P16185PC00 KJS/ALJ/SXH	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International application No. **PCT/AU 98/00648**	International filing date *(day/month/year)* 14 August 1998	Priority Date *(day/month/year)* 14 August 1997

International Patent Classification (IPC) or national classification and IPC

Int. Cl.[6] C12N 15/63, 15/67, 15/86; A61K 39/235

Applicant

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION *et al*

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 42 **(21 pages of description, 5 pages of claims, 15 sheets of drawings and a page of abstract)** sheet(s).

3. This report contains indications relating to the following items:

I	[X]	Basis of the report
II	[ ]	Priority
III	[ ]	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	[ ]	Lack of unity of invention
V	[X]	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	[ ]	Certain documents cited
VII	[ ]	Certain defects in the international application
VIII	[X]	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
10 March 1999	26 November 1999
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (02) 6285 3929	Authorized Officer **J.H. CHAN** Telephone No. (02) 6283 2340

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/AU 98/00648

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed.

☒ the description, pages , as originally filed,
pages , filed with the demand,
pages **1-21** filed with the letter of **11 November 1999**.

☒ the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages **22-26** , filed with the letter of **11 November 1999**.

☒ the drawings, pages , as originally filed,
pages , filed with the demand,
figures/sheets 1/15-15/15, filed with the letter of **11 November 1999**.

☐ the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages

☐ the claims, Nos.

☐ the drawings, sheets/fig.

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	**YES**
	Claims **1-42**	**NO**
Inventive step (IS)	Claims	**YES**
	Claims **1-42**	**NO**
Industrial applicability (IA)	Claims **1-42**	**YES**
	Claims	**NO**

2. Citations and explanations (Rule 70.7)

The abbreviations D1-D10 refer to the documents in the order as they appear in the international search report.

Novelty and inventive step:

The closest prior art are found in documents D7 (Reddy et al 1995) and D8 (Reddy et al 1996). Each of these documents discloses a recombinant porcine adenovirus wherein the E3 region of the adenoviral genome is suggested as being an appropriate site for insertion of a heterologous DNA. D7 contemplates PAV-3 as a vector for foreign genes in swine and D8, the PAV 1-3 as expression vectors. Whilst accepting the applicants' submission that there is no disclosure of any porcine adenovirus constructs in the documents, various controlling elements associated with the E3 regions eg the GC box, the CAAT box and the TATA box, have been disclosed therein. (See for example, page 108 of D8 and page 100 of D7). Such disclosures would enable the skilled addressee to construct a recombinant vector for expression of foreign gene based on PAV 1-3 with high expectation of success. Since the applicants have not stated that there were some unexpected practical difficulties associated with the preparation of such constructs, the invention as defined in claims 1-42 would not be novel and deprived of an inventive merit in the light of the disclosures in documents D7 and D8.

Industrial applicability:

The invention as defined in claims 1-42 is deemed to have industrial applicability.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

(a) Claims 1-28, 31-42 are not fully support by the description.
The description discloses the production of a recombinant porcine adenovirus wherein the whole or part of E 3 region of the adenoviral genome is deleted and replaced with a heterologous DNA sequence of interest. Since the "appropriate site" for the integration into the genome has not been defined in these claims, the scope of these claims includes any site for insertion on the porcine adenoviral genome. The applicants' submissions have stated that at the priority date of the current application, that porcine adenoviruses had not been examined in great detail and that little work had been published on the characterisation of the genome. Consequently, claims to the recombinant porcine adenovirus comprising a DNA of interest which has been stably integrated in any appropriate site on the genome, cannot be fully supported by the description.

(b) The description discloses the state of prior art using the specific format; ie "(name of the author and the year of publication of the article)". Such a format requires a list of bibliography to fully describe these articles. However there is no such list in the specification on file. For this reason the description relating to the prior art is not clear.

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RECOMBINANT PORCINE ADENOVIRUS VECTOR

FIELD OF INVENTION

This invention relates to delivery vectors for antigen producing genes (heterologous gene sequences or fragments thereof) used to generate immune responses in commercial pigs susceptible to decimation by disease. Such vectors are especially useful for the preparation of vaccines which can be easily administered on a large scale to protect pigs against disease. This invention also relates to a method of production of suitable delivery vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to a method protecting pigs from disease.

BACKGROUND

The productivity of the intensive pig industry depends on the control of infectious diseases. Whilst diseases can be controlled in part by good hygiene and quarantine measures, the industry must still rely on vaccination to protect herds. In a commercial situation, the cost per animal is high in terms of feed and current disease control costs and therefore, the costs in disease prevention and control by any newly proposed vaccine must be cheap, effective and easy to deliver.

Conventionally, vaccines constituting live viral particles have been prepared by virus passage and selection of attenuated forms. Alternatively, killed vaccines were prepared from virulent viruses.

The most recent description of the use of viral vectors in the control of disease in pigs was the deletion mutant of pseudorabies virus for the control of Aujesky's disease. The use of a herpesvirus as a vector has the advantage of being able to stimulate a humoral and cell-mediated response, thus providing possible life long protection. Another advantage is the ability to insert other heterologous sequences in this vector, being expressed from a suitable promoter, to produce antigens for exposure to the animals immune system, thus protecting against two diseases. There are disadvantages of this system. Firstly, there is the issue of latency. Herpesviruses have the ability to intergrate into the neurons in ganglia for the life of the animal. It only requires a suitable stress on the animal to cause the reactivation of the virus and consequently full disease. However, it is now known that the deletion of a specific gene, glycoprotein E, will attenuate the virus and prevent reactivation from latency. Therefore, this deletion vector is now widely used as an eradication vector for Aujesky's disease and subsequently will not be available as a suitable vector for the delivery of other antigens.

It is thus the aim of this invention to provide a delivery vehicle for heterologous sequences of genetic material that is particularly suited to administration on a large scale.

In particular, it is the aim of this invention to provide or enhance means for generation and/or optimisation of antibodies or cell-mediated immunity so as to provide protection against infection with common porcine diseases. It is an additional aim to provide a process for preparation of a suitable means for generation and/or optimisation of antibodies or cell-mediated immunity so as to protect pigs

against infection with common porcine diseases. It is a further aim to provide a protection strategy.

SUMMARY OF INVENTION

The invention provides, in one embodiment, a recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine adenovirus genome.

In another embodiment the invention provides a recombinant vector including a recombinant porcine adenovirus which stably incorporates at least one heterologous nucleotide sequence. Preferably the heterologous nucleotide sequence is capable of expression as an antigenic polypeptide. The antigenic polypeptide encoded by at least one nucleotide sequence is preferably foreign to the host vector.

In a further embodiment of the present invention the heterologous nucleotide sequence is capable of expression as an immuno-potentiator molecule.

It is also to be understood that the heterologous nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

The recombinant vector may comprise a live recombinant porcine adenovirus in which the virion structural proteins are unchanged from those in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

This invention is partially predicated on the discovery that there are non-essential regions in the porcine adenovirus genome which do not correspond to those characterised previously on other adenoviruses thus making this virus particularly suited to delivery of heterologous sequences.

This invention is also predicated on the discovery that the porcine adenovirus generates a prolonged response in pigs thus making it well suited as a vaccine vehicle. Furthermore, the existence of a number of serotypes specific to respiratory or gastrointestinal tracts, allows the selection of a vaccine vehicle suited to a target organ and the type of immune response required.

The invention is also predicated on the discovery that porcine adenovirus can package genomic DNA greater than the 105% rule for mammalian adenoviruses with intermediate size genomes and that the resultant packaged virions are stable in vitro and in vivo.

Adenoviruses are a large and diverse family, having been isolated from many living species, including man and other mammals as well as a variety of birds. As a result adenoviruses have been separated into at least two genera, the *Mastoadenoviridae* and the *Aviadenoviridae*, and more recently a third genera has been proposed, the *Atadenovirdae*, which includes some bovine and avian adenoviruses (egg drop syndrome) (Benkö and Harrach, Archives of Virology 143, 829-837, 1998).

Porcine adenoviruses are prevalent infectious agents of pigs and to date four distinct serotypes have been recognised (Adair and McFerran, 1976) and evidence for at least one more (Derbyshire *et*

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al., 1975). Of the four serotypes found, three (serotypes 1 to 3) were isolated from the gastrointestinal tract while the fourth was recovered from the respiratory system. The porcine adenoviruses are considered to be a low pathogenic widespread agent and although isolations were made in general from diseased animals, it was most likely that the adenovirus was present only as a secondary infection. They have been isolated from pigs with diarrhoea and respiratory infections but it has been considered that at least the gastrointestinal adenovirus infections are usually asymptomatic (Sanford and Hoover, 1983). Porcine adenoviruses are spread by ingestion or inhalation and experimental infection via oral, intranasal and intratracheal inoculations have resulted in uptake of the virus. Experimental pathogenicity studies have shown that the primary sites of infection are the lower small intestine probably the tonsil (Sharpe and Jessett, 1967; Shadduck *et al.*, 1968). With serotype 4 infection, a viraemia appears to develop in experimental infections. However, this may be a less common manifestation with the gastrointestinal serotypes (Shadduck *et al.*, 1968). Faecal excretion is the most common cause for spread of PAV, being present for several weeks post infection. Nasal shedding also occurs under experimental conditions. PAV's role in pneumonia has been suggested to be that of either a predisposing factor or a synergist (Kasza *et al.*, 1969; Schiefer *et al.*, 1974) but experimental pneumonia with serotype 4 did not require a second agent to produce disease (Smith *et al.*,1973).

Porcine adenoviruses have yet to be examined in much detail and little is known about their role in disease or how common they are. This is due to the fact that they do not produce any significant disease in herds and have failed to draw the interest of industry through loss of production. It is likely that the number of serotypes of porcine adenoviruses is much greater than four and that it probably exists in the majority of pig herds as a normal commensal.

Work done on porcine adenovirus in regards to its morphology and molecular biology, has shown some similarities with other *Mastadenoviruses* examined. Its morphology is that of other adenoviruses examined with an icosahedral capsid containing a core of a double stranded DNA genome. Very little work on the characterisation of the PAV genome has been published (Benkö *et al.*, 1990, Kleiboeker *et al.*, 1993, Reddy *et al.*, 1993, Kleiboeker, 1994). The size of the PAV genome (approx. 34.8 kb) is slightly smaller than that of human adenoviruses (approx. 35.9 kb). One study has shown using hybridisation with DNA probes from the total genome of human adenovirus type 2 that there is reasonable DNA homology between the porcine and human adenoviruses (Benkö *et al.*, 1990). A recent report on the serotype 4 PAV demonstrated that its genomic layout was also similar to that of the human adenoviruses in the area of the L4 and E3 regions (including the 33K and pVIII genes) even though the sequence homology was not as strong as may have been expected (Kleiboeker, 1994).

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While choosing appropriate PAV for development as a live vectors to deliver vaccines to pigs, it is important to take into account the natural prevalence of serotypes. Those serotypes not commonly encountered in the field have an obvious advantages over those to which pigs are frequently exposed and to which they may have developed immunity.

A further consideration is the ability of the vector to remain active in the pig beyond the period which maternal antibodies in colostrum protect pigs immediately post-birth.

Other important considerations in choosing potential PAV vectors are pathogenicity and immunogenicity. Preferably live vector viruses should be highly infectious but non-pathogenic (or at least attenuated) such that they do not themselves adversely affect the target species.

The preferred candidates for vaccine vectors are non-pathogenic isolates of serotype 4 (respiratory) and serotype 3 (gastrointestinal). Serotype 3 has been chosen as the serotype of choice due to excellent growth abilities in continuous pig kidney cell lines. The isolation of other serotypes, which seems likely, may well alter this selection. It is notable that the more virulent strains produce a greater antibody response.

Heterologous nucleotide sequences which may be incorporated into non-essential regions of the viral genome and which may encode the antigenic determinants of infectious organisms against which the generation of antibodies or cell-mediated immunity is desirable may be those expressing antigenic determinants of intestinal infections caused by gastrointestinal viruses; for example rotavirus or parvovirus infections, or respiratory viruses, for example parainfluenza virus, or that of Japanese encephalitis.

Heterologous nucleotide sequences which may be incorporated include the antigenic determinants of the agents of:

- Porcine parvovirus
- Mycoplasma hyopneumonia
- Porcine parainfluenza
- Transmissable gastroenteritis (porcine coronavirus)
- Porcine rotavirus
- Hog cholera virus (Classical swine fever)
- Swine dysentery
- African swine fever virus
- Pseudorabies virus (Aujesky's disease virus) in particular, the glycoprotein D of the pseudorabies virus
- Porcine respiratory and reproductive syndrome virus (PRRSV)

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- Heterologous nucleotide sequences more preferred for incorporation in the vectors of the invention are those expressing antigenic determinants of porcine parvovirus, porcine rotavirus, porcine coronavirus and classical swine fever virus.

It is also envisaged the heterologous sequences incorporated may be immuno-potentiator molecules such as cytokines or growth promoters, for example porcine interleukin 4 (IL4), gamma interferon (γIFN), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), FLT-3 ligand and interleukin 3 (IL-3).

The type of immune response stimulated by candidate vectors may affect the selection of heterologous nucleotide sequences for insertion therein. PAV serotypes 1, 2 and 3, which naturally infect via the gut may induce local mucosal immunity and are thus more suitable for infections of the intestines (eg classical swine fever virus). PAV serotype 4, which naturally infects via the respiratory system, may be more suitable for infections of the respiratory tract (eg porcine parainfluenza) may also induce good local immunity.

The DNA of interest which may comprise heterologous genes coding for antigenic determinants or immuno-potentiator molecules may be located in at least one non-essential region of the viral genome.

Non-essential regions of the viral genome which may be suitable for the purposes of replacement with or insertion of heterologous nucleotide sequences may be non-coding regions at the right terminal end of the genome at map units 97 to 99.5. Preferred non-coding regions include the early region (E3) of the PAV genome at map units 81-84.

The heterologous gene sequences may be associated with a promoter and leader sequence in order that the nucleotide sequence may be expressed in situ as efficiently as possible. Preferably the heterologous gene sequence is associated with the porcine adenoviral major late promoter and splice leader sequence. The mammalian adenovirus major late promoter lies near 16-17 map units on the adenovirus genetic map and contains a classical TATA sequence motif (Johnson, D.C., Ghosh-Chondhury, G., Smiley, J.R., Fallis, L. and Graham, F.L. (1988), Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology 164, 1-14).

The splice leader sequence of the porcine adenovirus serotype under consideration is a tripartite sequence spliced to the 5' end of the mRNA of all late genes.

The heterologous gene sequence may also be associated with a poly adenylation sequence.

Instead of the porcine adenoviral major late promoter, any other suitable eukaryotic promoter may be used. For example, those of SV40 virus, cytomegalovirus (CMV) or human adenovirus may be used.

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Processing and poly adenylation signals other than those of porcine adenoviruses may also be considered, for example, that of SV40.

In a further aspect of the invention there is provided a recombinant vaccine for generating and/or optimising antibodies or cell-mediated immunity so as to provide or enhance protection against infection with an infectious organism in pigs, the vaccine including at least one recombinant porcine adenovirus vector stably incorporating at least one heterologous nucleotide sequence formulated with suitable carriers and excipients. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide or as an immuno-potentiator molecule. More preferably, the heterologous nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

The antigenic polypeptide encoded by the at least one nucleotide sequence is preferably foreign to the host vector. At least one nucleotide sequence may be associated with a promoter/leader and a poly A sequence.

The recombinant vaccine may include live recombinant porcine adenovirus vector in which the virion structural proteins are unchanged from that in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

Preferred vector candidates for use in the recombinant vaccine are PAV isolates of serotype 3 and 4. Use of other serotypes is possible, depending on herd existing immunity and its environment.

The vaccine may be directed against respiratory and intestinal infections caused by a variety of agents. In order to direct the vaccine against a specific infectious organism, heterologous gene sequences encoding the antigenic determinants of those infectious organisms may be incorporated into non-essential regions of the genome of the porcine adenovirus comprising the vector. If the vaccine is to be used to optimise protection against disease, suitable heterologous nucleotide sequences may be those of immuno-potentiators such as cytokines or growth promoters.

The vaccine may comprise other constituents, such as stabilisers, excipients, other pharmaceutically acceptable compounds or any other antigen or part thereof. The vaccine may be in the form of a lyophilised preparation or as a suspension, all of which are common in the field of vaccine production.

A suitable carrier for such as a vaccine may be isotonic buffered saline.

In a further aspect of the invention, there is provided a method of preparing a vaccine for generation and/or optimisation of antibodies or cell-mediated immunity so as to induce or enhance protection against an infectious organism in a pig, which includes constructing a recombinant porcine adenovirus vector stably incorporating at least one heterologous nucleotide sequence, and placing said

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recombinant porcine adenovirus vector in a form suitable for administration. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide although it may also be an immuno-potentiator molecule. More preferably, the nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiatormolecule. The nucleotide sequence is conveniently foreign to the host vector.

Even more preferably, the nucleotide sequence is associated with promoter/leader and poly A sequences.

The form of administration may be that of an enteric coated dosage unit, an inoculum for intra-peritoneal, intramuscular or subcutaneous administration, an aerosol spray, by oral or intranasal application. Administration in the drinking water or in feed pellets is also possible.

In another aspect of the invention, there is provided a method of producing a porcine adenovirus vaccine vector which includes inserting into a porcine adenovirus at least one heterologous nucleotide sequence. Said heterologous nucleotide sequence is preferably capable of expression as an antigenic polypeptide although it may also be an immuno-potentiator molecule. More preferably, the nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

Preferably the antigenic polypeptide encoded by the at least one nucleotide sequence is foreign to the host vector.

More preferably, the heterologous nucleotide sequence is associated with promoter/leader and poly A sequences.

In one method of construction of a suitable vector the non-essential region to be altered to incorporate foreign DNA could be constructed via homologous recombination. By this method the non-essential region is cloned and foreign DNA together with promoter, leader and poly adenylation sequences is inserted preferably by homologous recombination between flanking sequences. By this method also, deletion of portions of the non-essential region is possible to create extra room for larger DNA inserts that are beyond the normal packing constraints of the virus.

By this method a DNA expression cassette containing an appropriate PAV promoter with foreign gene sequence as well as leader sequences and poly adenylation recognition sequences can be constructed with the unique restriction enzyme sites flanking the cassette enabling easy insertion into the PAV genome.

In another aspect of the invention there is provided strategies for administration of the vaccines of the invention.

In one strategy, a heterologous antigen and immuno-modulatory molecule such as a cytokine may be expressed in the same recombinant and delivered as a single vaccine.

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In one strategy according to the invention PAV vector based vaccines may be administered as 'cocktails' comprising 2 or more virus vectors carrying different foreign genes or immuno-potentiators.

In a preferred vaccination strategy of the invention, the 'cocktail' or simultaneous strategy, a vaccine based on both PAV serotype 3 and serotype 4 is used.

In another preferred strategy, a base recombinant serotype 3 porcine adenovirus is constructed and the fiber gene from serotype 4 replacing that of serotype 3 or the fiber from serotype 4 additionally cloned into the vaccine to broaden the targeting of the invention to both gut and respiratory delivery.

In an alternative strategy according to the invention, PAV vector based vaccines may be administered consecutively of each other to either administer booster vaccines or new vaccines at some stage subsequent to initial PAV vaccination. The vaccines used are preferably based on heterologous PAV isolates.

In a preferred version of the "consecutive" strategy, vaccines based on isolates serotypically unrelated are selected so as to achieve maximum protection against infection. In one example of such a strategy a vaccine based on PAV serotype 3 is administered subsequently or prior to vaccination with a vaccine based on PAV serotype 4.

Pigs are conveniently inoculated with vector vaccines according to the invention at any age. Piglets may be vaccinated at 1 day old, breeders may be vaccinated regularly up to point of giving birth and thereafter.

Preferably according to either the consecutive strategy or the cocktail strategy, pigs are vaccinated while still not fully immunocompetent. More conveniently, day-old pigs can be vaccinated for protection against re-infection after a period of 4 weeks subsequent to initial vaccination.

In a further embodiment of the invention there is provided a method for producing an immune response in a pig including administering to the pig an effective amount of a recombinant vaccine according to the invention. An effective amount is an amount sufficient to elicit an immune response, preferably at least 10^4 $TCID_{50}$ per dose.

The vaccine of the invention may of course be combined with vaccines against other viruses or organisms such as parvovirus or Aujeszky's disease at the time of its administration.

In a preferred aspect of this embodiment of the invention, administration is by oral delivery or intra-nasally.

Methods for construction and testing of recombinant vectors and vaccines according to this invention will be well known to those skilled in the art. Standard procedures for endonuclease

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digestion, ligation and electrophoresis were carried out in accordance with the manufacturer's or suppliers instructions. Standard techniques are not described in detail and will be well understood by persons skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the DNA restriction endonuclease map of the entire PAV serotype 3 genome.

Figure 2 illustrates the sequence characterisation and cloning of the major later promoter and splice leader sequences of PAV serotype 3.

Figure 3 illustrates the sequences of the major later promoter, upstream enhancer sequence and splice leaders 1, 2 and 3.

Figure 4 illustrates the terminal 720 bases of the right end of the genome.

Figure 5 illustrates the promoter region of E3 and the overlapping L4 area.

Figure 6 illustrates a preferred method of construction of a PAV vector.

Figure 7 represents temperature data of pigs vaccinated with a PAV based vaccine following challenge with CSFV antigen.

Figure 8 graphically represents anti-PAV antibody levels detected by ELISA in pigs pre and post vaccination with a PAV based vaccine.

Figure 9 graphically illustrates the development of neutralising antibodies in pigs vaccinated with a PAV based vaccine pre and post challenge with CSFV antigen.

Figure 10 graphically illustrates the mean white blood cell (WBC) counts of pigs vaccinated with a recombinant PAV vaccine expressing porcine G-CSF.

Figure 11 graphically illustrates the percentage change in white blood cell (WBC) counts following vaccination with a recombinant PAV vaccine expressing porcine G-CSF.

Figure 12 graphically represents the percentage change in monocyte cell populations following vaccination with recombinant PAV-G-CSF.

Figure 13 graphically represents the percentage change in lymphocyte cell populations following vaccination with recombinant PAV-G-CSF.

Figure 14 graphically represents the change in stimulation of T-cells following vaccination with recombinant PAV-G-CSF.

Figure 15 a, b and c graphically illustrate a method of construction of a PAV E3 vector.

PREFERRED EMBODIMENTS

Aspects of preferred embodiments of the invention based on PAV isolates serotype 3 and serotype 4 will now be described. Whilst these two isolates have been selected because of their sites of infection in the pig, it will be appreciated that other isolates of porcine adenovirus may be more suitable for construction of vaccine vectors provided the criteria for selection described herein before

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are met.

In general, PAV are considered of low pathogenicity with little consequence in the field. The pathogenic significance of PAV is reviewed in Derbyshire, 1989. The first report of isolation of PAV was from a 12 day old pig with diarrhoea (Haig *et al.*, 1964). Two years later, PAV type 4 was first reported, isolated from the brain of a pig suffering from encephalitis of unknown cause (Kasza, 1966). Later reports have associated PAV mainly with diarrhoea in the field although this is normally low grade. PAV can also be regularly isolated from healthy animals with no disease signs and it is quite likely that its isolation from diseased animals is more a coincidence of its prevalence than an indicator of pathogenicity. However, an association between serotype 4 and respiratory disease has been reported (Watt, 1978) and this has been supported by experimental infection (Edington *et al.*, 1972). Experimental infections with gastrointestinal serotypes of the virus (eg serotype 3) have been able to produce diarrhoea but the pathological changes produced were not clinically significant.

The genome of the selected PAV serotype 3 was characterised by conventional methods. The DNA restriction endonuclease maps of the entire genome is illustrated in figure 1. The genomes are orientated left to right. By convention adenovirus genomes are normally orientated such that the terminal region from which no late mRNA transcripts are synthesised is located at the left end. The enzymes used to generate the map are indicated at the edge of each map.

CHARACTERISATION OF MAJOR LATE PROMOTER (MLP) AND SPLICE LEADER SEQUENCES (LS) OF PAV SEROTYPE 3

Identification and cloning of the PAV MLP

By use of restriction enzyme and genetic maps of the PAV serotype 3 genome, a region was located that contained the MLP and leader sequences (Fig 1). The fragments identified in this region were cloned into plasmid vectors and sequenced.

The MLP promoter sequence was identified as containing a classical TATA sequence, the only one in the region sequenced, as well as upstream factors and was subsequently confirmed by the location of the leader sequence and the transcriptional start site.

Figures 2 and 3 illustrate the sequence characterisation of the major late promoter and splice leader sequences of PAV serotype 3.

In order to determine the structure and sequence of the leader sequence spliced to late mRNA, porcine kidney cells were infected with PAV and the infection was allowed to proceed until late in the infection cycle (usually 20-24 hr p.i.). At this time total RNA was purified from the infected cells using the RNAgents total RNA purification kit (Promega). The isolated RNA was precipitated with isopropanol and stored at -70°C in 200 µl aliquots until required. Poly A (mRNA) was isolated from total RNA by the use of the Poly AT tract System (Promega, USA). The isolated mRNA was used in cDNA

production.

For cDNA production, oligonucleotides were produced to the complimentary strand of the hexon gene and the penton base gene, both being MLP transcripts. A further oligonucleotide was produced which covered the proposed cap site of the major late transcript, 24 bases downstream of the TATA box. This oligonucleotide was used in conjunction with that used in cDNA production in Taq polymerase chain reaction. The resulting DNA produced from positive clones was digested with appropriate restriction enzymes to determine the size of the inserted fragment. DNA sequencing of these inserted fragments was performed using a modification of the chain termination technique (Sanger, F., Nicklen, S and Gulson, A.R., 1977, DNA sequencing with chain terminating inhibitors. PNAS USA 74: 5463-5467) so as to allow Taq DNA polymerase extension (Promega, USA).

To confirm the leader sequence cap site, fresh cDNA was prepared and this time a tail of dGTP residues added to it. Briefly, cDNA was incubated with 1 mM dGTP and approximately 15 units of terminal deoxynucleotidyl transferase (Promega) in 2 mM CaCl2 buffer at 37°C for 60 minutes. The reaction was stopped by heating to 70°C for 10 minutes. The DNA was then ethanol precipitated and resuspended in a volume suitable for use in polymerase chain reaction (PCR). PCR was performed as previously described using a poly (dC) oligonucleotide with a *Xba*I site at the 5' end. Resulting fragments were blunt ended with T4 DNA polymerase at 37°C for 30 minutes in the presence of excess nucleotides and cloned into the *Sma*I site of the pUC18 vector. DNA preparation and sequencing were performed, as described previously, on clones shown to be positive by hybridisation.

Figure 3 illustrates the separate sequences of the major late promoter, upstream enhancer sequence and splice leaders 1, 2 and 3 as determined from cDNA studies. Figure 2 illustrates the DNA sequence of the complete promoter cassette with the components joined together.

CHARACTERISATION OF NON-ESSENTIAL REGIONS OF VIRAL GENOME

The right end was identified by cloning and complete sequencing of the PAV serotype 3 *Apa*I fragment J of approximately 1.8 Kbp. The inverted terminal repeat (ITR) has been determined by comparison of the RHE sequence with that of the left hand end. The ITR is 144 bases long and represents the starting point into which potential insertions can be made. Figure 4 shows the sequence of the terminal 720 bases. Restriction endonuclease sites of interest for insertion of foreign DNA are indicated in the terminal sequence. A putative TATA site for the E4 promoter is identified, this being the left most end for the possible site of insertion. Initial insertions will be made into the *Sma*I or *Eco*RI sites.

The E3 region of the genome, this also being a non-essential area, has been located and cloned. The promoter region of E3 has been identified and the overlapping L4 area sequenced (Figure 5). The region of the E3 after the polyadenylation signal of the L4 is also a possible site for insertion

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and can also be used for deletion to create more room for larger cassette insertions.

CONSTRUCTION OF PAV VECTOR

Figure 6 illustrates a preferred method of construction of a PAV vector. The right hand end *Apa*I fragment J of PAV serotype 3 is cloned and a unique *Sma*I restriction endonuclease site 230 bp from the inverted repeats was used as an insertion site.

The major late promoter expression cassette containing the E2 (gp55) gene of classical swine fever virus (hog cholera virus) was cloned into the *Sma*I site of the RHE fragment.

A preferred method of homologous recombination was cutting genomic PAV 3 DNA with *Hpa*I, a unique site in the genome, and transfecting this DNA with *Apa*I cut expression cassette plasmid containing gp55.

The DNA mix was transfected into preferably primary pig kidney cells by standard calcium chloride precipitation techniques.

The preferred method of transfection generates recombinant virus through homologous recombination between genomic PAV 3 and plasmid (Fig 6).

DETAILED DESCRIPTION OF THE INVENTION

CONSTRUCTION OF PAV VECTOR

The following examples show the construction of representative recombinant porcine adenoviruses of this invention. The recombinant viruses were propagated and titred on primary porcine kidney cells.

1 Construction of PAV-gp55

An expression cassette consisting of the porcine adenovirus major late promoter, the classical swine fever virus (CSFV) gene (gp55) and SV 40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.38 kilobase pairs. No deletion of the genomic PAV 3 was made. Mammalian adenoviruses with intermediate genomes (~36kb) have been shown to accommodate up to 105% of the wild-type genomic length, and genomes larger than this size are either unpackageable or extremely unstable, frequently undergoing DNA rearrangements (Betts, Prevec and Graham, Journal of Virology 67, 5911-5921 (1993), Packaging capacity and stability of human adenovirus type 5 vectors: Parks and Graham, Journal of Virology, 71, 3293-3298, (1997), A helper dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging). In this invention, PAV genomic length was 34.8 kb, into which was inserted without any other deletion an expression cassette of 2.38 kb. The resulting genomic DNA length of the recombinant porcine adenovirus of this invention was 106.8%, and therefore exceeded the putative maximum limit for construction of a stable recombinant. The recombinant virus was plaque purified three times and passaged stably in primary pig kidney cells. The recombinant was shown to

contain gp55 by Southern blot hybridisation. Expression of gp55 was demonstrated by infecting primary PK cell line grown on glass cover slips with the recombinant porcine adenovirus. After 24 hours, immunoflouresencent staining (IF) showed infected cells expressing gp55.

2 Construction of recombinant PAV-G-CSF

An expression cassette comprising of the porcine adenovirus major late promoter, the gene encoding porcine granulocyte-colony stimulating factor (G-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 1.28 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and passaged stably in primary pig kidney cells. The recombinant was shown to contain G-CSF by Southern blot hybridisation and polymerase chain reaction (PCR). Expression of G-CSF was demonstrated by infecting primary kidney cells with the recombinant PAV-G-CSF. Tissue culture supernatants from the infected primary kidney cells were then electrophoresed in SDS-PAGE gels and transferred to filters. Infected cells expressing G-CSF were detected in a Western blot using a rabbit polyclonal antiserum against porcine G-CSF expressed by purified recombinant *E coli*.

3 Construction of recombinant PAV-gp55T/GM-CSF

An expression cassette consisting of the porcine adenovirus major late promoter, a truncated form of the classical swine fever virus gene gp55 fused in frame to the gene encoding either the full length or the mature form of porcine granulocyte/macrophage-colony stimulating factor (GM-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.1 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and shown to contain gp55 and GM-CSF by PCR.

4 Construction of Recombinant PAV-gp55/E3

The insertion vector pJJ408 containing the right hand end *ApaI* fragment J of the PAV serotype 3 genome (approximately 1.8kbp), was enlarged to contain the complete *Bgl*II B fragment comprising 7.2kbp of the PAV3 right hand end (Figure 15a and b). This fragment contains both the right hand end insertion site described previously and the E3 region. The right hand end insertion site was engineered to contain the PAV3 MLP/TPL sequences followed by a multiple cloning site and the SV40 poly A sequence.

An E3 insertion site was constructed by excising a 622bp *SnaBI/BsrGI* fragment within the E3 region of the PAV serotype 3. The MLP/TPL-gp55-Poly A expression cassette was inserted into the *SnaBI/BsrGI* site (Figure 15b and c). This plasmid was used in transfections to produce a recombinant

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PAV3 containing the MLP/TPL-gp55-poly A cassette inserted in the partially deleted E3 region (Figure 15c).

Wild type PAV3 DNA was digested with *SnaB*I restriction enzyme yielding two fragments of 28.712 kbp and 5.382 kbp. The large left hand fragment which includes the overlap region of the right hand end and the left hand end of the PAV3 genome was gel purified. This fragment was transfected into primary PK cells along with *Kpn*I restricted E3/rhe insertion vector DNA in 3cm petri dishes to allow homologous recombination to occur between the PAV3 and insertion vector DNA. Using this method, only recombinant virus are recovered.

Cells were maintained for 5 days at 37°C and then frozen and thawed twice. Lysate was passaged into fresh primary PK cells and observed for the development of plaques. The recombinant virus was plaqued purified and shown to contain gp55 by PCR.

VACCINATION STRATEGY

1. Vaccination with PAV-gp55

In this experiment 5-6 week old piglets were used to represent immunocompetent pigs. A group of the piglets (#2, 6 and 7) were vaccinated with recombinant PAV-gp55 administered subcutaneously at a dose of 1 X 10^7 pfu per piglet. A control group of piglets (#3, 8, 11, 12, 13 and 14) were unvaccinated. No clinical signs were observed (no rise in temperature) in the vaccinated group of piglets (Table 1).

Temperatures post Vaccination with rPAV:: gp55 CSFV (°C)								
Pig No.	**Day 0**	**1**	**2**	**3**	**6**	**9**	**10**	**13**
2	39.7	39.2	39.4	39.8	39.6	39.8	39.6	39.2
3 (control)	39.5	39.2	39.4	39.0	38.8	39.3	39.0	39.7
6	39.7	39.1	39.1	39.0	39.1	39.8	39.1	39.8
7	39.4	39.8	39.8	39.4	39.9	38.9	39.6	39.7
8 (control)	39.6	39.5	39.4	39.0	40.5	39.4	39.1	39.7

Five weeks after vaccination with the recombinant PAV-gp55 both groups of pigs were challenged with a lethal dose (1 X $10^{3.5}$ $TCID_{50}$) of virulent Hog Cholera virus (Classical swine fever virus) applied subcutaneously.

The temperatures of the pigs were monitored and the results tabulated in Table 2 and graphically represented in Figure 7.

Table 2: Temperatures post challenge with CSFV (°C)

Pig No.	Day	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2		39.6	39.9	40.1	40.3	40.1	39.2	39.7	39.5	39.5	39.7	39.4	39.1	39.5	39.1	40.0	39.4	38.9
3		39.6	40.4	39.6	40.0	40.7	40.7	41.9	40.7	40.9	42.0+							
6		39.5	39.5	40.0	40.0	39.6	40.5	39.9	39.2	39.2	38.8	39.3	38.8	38.9	39.6	39.3	39.1	[redacted]
7		39.8	39.9	40.4	40.6	40.3	39.7	39.7	39.7	39.5	39.3	39.1	39.3	39.6	40.6	39.7	39.8	39.7
8		39.9	40.6	40.5	40.3	40.0	41.4	39.8	41.0	40.6	39.0+							
11		39.6	39.9	40.0	40.3	40.7	40.5	40.0	41.8	41.5	41.3+							
12		39.8	39.9	40.9	41.0	41.2	40.6	40.1	41.0	41.7	40.3+							
13		39.7	40.0	41.2	41.5	41.6	41.0	39.7+										
14		39.3	40.0	39.6	39.8	40.3	40.7	41.2	40.8	40.2	41.7+							

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The results show that by day 5 the control group had elevated temperatures (greater than 40.5°C) and showed clinical signs of disease. The vaccinated group showed no clinical signs of disease. Pigs from the control group were dead or euthanased by day 9. The vaccinated group were euthanased at day 16. At post mortem all control pigs showed severe clinical disease, the vaccinated pigs showed no clinical signs of disease.

The results indicate that the pigs vaccinated subcutaneously with the recombinant PAV-gp55 survived challenge with classical swine fever virus at a lethal dose.

Sera were collected from both groups of pigs and tested for the presence of antibodies to PAV by ELISA. These tests showed the presence of pre-existing antibodies to PAV before vaccination. The level of these antibodies increased following vaccination with the recombinant PAV-gp55 to peak between days 28 and 36 post vaccination. These results are tabulated in Figure 8.

Sera were collected from the vaccinated group of pigs pre and post challenge with CSFV and tested in the presence of neutralising antibodies to CSFV. Sera were tested at days 0 and 28 after vaccination with recombinant PAV-gp55 (pre challenge) and then again at day 16 post challenge (day 52 after vaccination). The results in Figure 9 show no neutralising antibodies detected at day 0, low levels of neutralising antibodies at day 28 and high levels at day 52.

These results show that the recombinant PAV-gp55 can protect pigs from lethal challenge with classical swine fever virus in the presence of pre-existing antibodies to PAV.

2. Vaccination with PAV-G-CSF

In this experiment 5-6 week old piglets were used to represent immunocompetent pigs. A group of pigs (n=4) were vaccinated with recombinant PAV-G-CSF administered subcutaneously at a dose of 1 X 10^7 pfu per piglet. A second group (n=4) were vaccinated with PAV wild type (wt) administered subcutaneously at a dose of 1 X 10^7 pfu per piglet. A control group (n=4) were unvaccinated. Pigs were bled at 8 hour intervals for a period of 104 hours post vaccination. Complete blood counts were determined and the mean white blood cell (WBC) counts for each group monitored. These results are graphically represented in Figure 10 and the percentage change in mean WBC counts graphically represented in Figure 11.

Pigs vaccinated with either PAV wt or PAV-G-CSF showed clinical signs of disease with mild diahorrea 24-72 hours post vaccination. Both groups of pigs were completely recovered by 80-96 hours post-vaccination. Control pigs showed no clinical signs of disease.

Complete blood screening results show that the mean WBC counts for control pigs increased over the duration of the experiment.

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PAV wt vaccinated pigs also show an increase in WBC counts, with a depression in WBC counts between 48-80 hours post-vaccination and recovery from 80-96 hours onwards.

Pigs vaccinated with the recombinant PAV-G-CSF show a significant depression in WBC counts over the duration of the experiment. A statistical analysis of these results is tabulated in Table 3. The analysis shows that differences between the mean WBC counts (controls and PAV-G-CSF; PAV wt and PAV-G-CSF) were significant indicating that the recombinant PAV-G-CSF altered the proportions of cells involved with immunity.

Table 3. Results of t-tests between mean WBC counts of groups of pigs vaccinated with either PAV wild type (wt), PAV recombinant expressing G-CSF (PAV-G-CSF) or unvaccinated controls.

	Pre vacc 0 hr	8-24 hr[d]	32-48 hr	56-72 hr	80-104 hr
Control vs PAV-G-CSF[a]	p> 0.2[b]	P> 0.2	P> 0.2	P> 0.2	P< 0.005
Control vs PAV wt	p> 0.1	p> 0.01[c]	p> 0.02	p> 0.2	P< 0.05
PAV-G-CSF Vs PAV wt	p> 0.2	p> 0.05	p> 0.05	P<0.05	P< 0.001

a: null hypothesis; there is no difference between the mean WBC counts.

b: p>0.05, insufficient to reject the null hypotheses at the 95% confidence level, conclude that there is no difference between mean leucocyte levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean leucocyte levels.

d: 4 pigs in each group were bled at 8 hour intervals.

Differential WBC counts were also determined and monitored for each group. The percentage change in mean monocyte cell populations graphically represented in Figure 12 and the percentage change in mean lymphocyte cell populations graphically represented in Figure 13. Figure 12 shows that monocyte cell populations increased rapidly in pigs following vaccination with PAV wt, but were suppressed by vaccination with the recombinant PAV-G-CSF. This effect was due to the expression of G-CSF by the recombinant. A statistical analysis of these results is tabulated in Table 4. The analysis shows that there was a significant difference between the PAV wt and PAV-G-CSF from 32 to 96 hours post vaccination. Figure 13 shows that there were shifts in lymphocyte cell population numbers following vaccination with the recombinant PAV-G-CSF. Unvaccinated controls show stable lymphocyte cell numbers over the duration of the experiment, whereas pigs vaccinated with PAV wt show a significant increase in lymphocyte cell population as a response to infection.

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Pigs vaccinated with the recombinant PAV-G-CSF show a decline in lymphocyte cell population. A statistical analysis of these results is tabulated in Table 5. The analysis shows that there was a significant difference between PAV wt and the recombinant PAV-G-CSF between 8 and 96 hours post vaccination. The different responses in lymphocyte cell proliferation following vaccination with recombinant PAV-G-CSF and PAV wt were due to the expression of G-CSF by the recombinant. These results show that vaccination with recombinant PAV-G-CSF produces a shift in sub-populations of cells involved in immunity.

Table 4. Results of t-tests between mean monocyte cell populations following vaccination of pigs with either recombinant PAV-G-CSF, wild type PAV (PAV wt) or unvaccinated controls.

	Pre vacc	8-24 hr[d]	32-48 hr	56-72 hr	80-96 hr	104 hr
Control Vs PAV-G-CSF[a]	p> 0.1[b]	P> 0.2	P> 0.2	P> 0.2	P>0.2	p> 0.2
Control Vs PAV wt	p> 0.2	P< 0.002[c]	p> 0.2	P< 0.001[c]	P> 0.2	p> 0.2
PAV wt Vs PAV-G-CSF	p> 0.2	P< 0.001	p> 0.2	P>0.2	P> 0.2	p> 0.05

a: null hypothesis; there is no difference between the mean monocyte cell counts.

b: p>0.1, insufficient to reject the null hypothesis at the 90% confidence level, conclude that there is no difference between mean monocyte cell levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean monocyte cell levels

d: 4 pigs in each group were bled at 8 hour intervals

Table 5. Results of t-tests between mean lymphocyte cell populations following vaccination of pigs with either recombinant PAV-G-CSF, wild type PAV (PAV wt) or unvaccinated controls.

	Pre vacc	8-24 hr[d]	32-48 hr	56-72 hr	80-96 hr	104 hr
Control Vs PAV-G-CSF[a]	p> 0.2	P> 0.05[b]	P> 0.2	P> 0.2	P>0.2	p> 0.2
Control Vs PAV wt	p> 0.2	P> 0.2	P< 0.01[c]	P< 0.001[c]	P< 0.001[c]	p> 0.2
PAV wt Vs PAV-G-CSF	p> 0.2	P< 0.05[c]	P< 0.002[c]	P< 0.005[c]	P< 0.001[c]	p> 0.05

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a: null hypothesis; there is no difference between the mean lymphocyte cell counts.

b: $p>0.05$, insufficient to reject the null hypothesis at the 95% confidence level, conclude that there is no difference between mean lymphocyte cell levels.

c: $p<0.05$, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean lymphocyte cell levels.

d: 4 pigs in each group were bled at 8 hour intervals

Figure 14 graphically represents changes in the proliferation of T cells of each group following stimulation with Concanavalin A (Con A). These results confirm that there was a significant proliferation of T-cells following vaccination with PAV wt at day 2 post vaccination, whereas vaccination with the recombinant PAV-G-CSF resulted in a suppression of T-cell proliferation by day 3.

The results of vaccination with a recombinant PAV expressing porcine G-CSF shows that G-CSF has a significant effect on the cells involved with immune responses.

It will be appreciated that whilst this document establishes the metes and bounds of this invention, all embodiments falling within its scope for example with regard to heterologous genes, insertion sites, types of promoter and serotype have not necessarily been specifically exemplified although it is intended that they should fall within the scope of protection afforded this invention.

Figure 2

Total sequence of the PAV Major Late Promoter cassette including the added nucleotides 5' (upstream) of the USF.

Nucleotide base count:76 A 143 C 187 G 96 T Total 502 bp

1	GGTGCCGCGG	TCGTCGGCGT	AGAGGATGAG	GGCCCAGTCG	GAGATGAAGG	CACGCGCCCA
61	GGCGAGGACG	AAGCTGGCGA	CCTGCGAGGG	GTAGCGGTCG	TTGGGCACTA	ATGGCGAGGC
121	CTGCTCGAGC	GTGTGGAGAC	AGAGGTCCTC	GTCGTCCGCG	TCCAGGAAGT	GGATTGGTCG
181	CCAGTGGTAG	**TCCACGTGAC**	CGGCTTGCGG	GTCGGGGGGT	**ATAAAA**GGCG	CGGGCCGGGG
241	TGCGTGGCCG	TC*A*GTTGCTT	*CGCAGGCCTC*	*GTCACCGGAG*	*TCCGCGTCTC*	*CGGCGTCTCG*
301	*CGCTGCGGCT*	*GCATCTGTGG*	*TCCCGGAGTC*	*TTCAGGTCCT*	*TGTTGAGGAG*	*GTACTCCTGA*
361	*TCGCTGTCCC*	*AGTACTTGGC*	*GTGTGGGAAG*	*CCGTCCTGAT*	*CGCGATCCTC*	*CTGCTGTTGC*
421	*AGCGCTTCGG*	*CAAACACGCG*	*CACCTGCTCT*	*TCGGACCCGG*	*CGAAGCGTTC*	*GACGAAGGCG*
481	*TCTAGCCAGC*	*AACAGTCGCA*	*AG*			

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The Upstream Stimulatory Factor (USF) and TATA motiff are in **bold**. The complete leader sequence is italised with the cap site and splice sites between the individual leaders indicated by double underlining or single underlining respectively.

Figure 3

Individual sequences of the Promoter cassette components:

I. The 5' (upstream) sequence included in the long cassette.

1	GGTGCCGCGG	TCGTCGGCGT	AGAGGATGAG	GGCCCAGTCG	GAGATGAAGG	CACGCGCCCA
61	GGCGAGGACG	AAGCTGGCGA	CCTGCGAGGG	GTAGCGGTCG	TTGGGCACTA	ATGGCGAGGC
121	CTGCTCGAGC	GTGTGGAGAC	AGAGGTCCTC	GTCGTCCGCG	TCCAGGAAGT	GGATTGGTCG
181	CCAGTGGTAG					

II. Sequence including the USF, TATA motiff and sequence to the cap site.

1	**CCACGTGACC**	GGCTTGCGGG	TCGGGGGGTA	**TAAAAGGCGC**	GGGCCGGGGT	GCGTGGCCGT
61	C					

III. First leader sequence.

1	AGTTGCTTCG	CAGGCCTCGT	CACCGGAGTC	CGCGTCTCCG	GCGTCTCGCG	CTGCGGCTGC
61	ATCTGTGGTC	CCGGAGTCTT	CAG			

IV. Second leader sequence.

1	GTCCTTGTTG	AGGAGGTACT	CCTGATCGCT	GTCCCAGTAC	TTGGCGTGTG	GGAAGCCGTC
61	CTGATCG					

V. Third leader sequence.

1	CGATCCTCCT	GCTGTTGCAG	CGCTTCGGCA	AACACGCGCA	CCTGCTCTTC	GGACCCGGCG
61	AAGCGTTCGA	CGAAGGCGTC	TAGCCAGCAA	CAGTCGCAAG		

Figure 4

Sequence of the right hand end of the PAV genome this area being a proposed site for insertion of expression cassettes.

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Nucleotide base count 183 A 255 C 306 G 204 T Total 948 bases

	1	**CATCATCAAT**	**AATATACCGC**	**ACACTTTTAT**	**TGCCCCTTTT**	**GTGGCGTGGT**	**GATTGGCGGA**
5	61	**GAGGGTTGGG**	**GGCGGCGGGC**	**GGTGATTGGT**	**GGAGAGGGGT**	**GTGACGTAGC**	**GTGGGAACGT**
	121	**GACGTCGCGT**	**GGGAAAATAA**	**CGTGGCGTGG**	GAACGGTCAA	AGTCCGAGGG	GCGGGGTCAA
	181	AGTCCGCAGT	CGCGGGGCGG	AGCCGGCTGG	CGGGAATTCC	CGGGACTTTC	TGGGCGGGTA
					EcoRI	SmaI	
	241	ATCGTTAACG	CGGAGGCGGG	GGAATTCCGA	TCGGACGATG	TGGTACTGAT	TAACCGACCG
10		HpaI		EcoRI			
	301	CAGGCGTGTC	CACATCCGCT	GTGGGTATAT	CACCGGCGCT	CGCGGTGTTC	GCTCACACTC
	361	GTCTCGGCGC	TGTCACAGAG	AGAGACACTG	AGAGCGAGAC	GAGGAGAAAC	CGAAAGCGGG
	421	GCAGGAGGAG	TCACCGGGCC	ATCTTCCCAT	CAGAGCCCTC	TCATGGCCCA	CGACCGACTG
	481	CTGCTGGCCG	CGGTGGCTGA	CTGTTGCTCG	CCGTGCTCTA	TCTGTACTTC	GCCTACCTCG
15	541	CGTGGCAGGA	TCGGGACACT	CTTCACACTC	AGGAGGCCGC	CTCTCCTCGC	TTCTTCATCG
	601	GGTCCAACCA	CCAGCCCTGG	TGCCCGGATT	TTGATTGGCA	GGAGCAGGAC	GAGCACACTC
	661	ACTAGACGTT	TAGAAAAAAG	ACACACATTG	GAACTCATAT	ATGTCTGCGG	GACCGCATCA
	721	GCAGCCCGGT	CTGCTGTTGG	CTGCGGGTGA	GAGGCCTCCG	GTAATTCATC	AGAACCGCAT
					StuI		
20	781	TCATCTGCGC	CACGTCCCGA	CATATGGTGC	TGACGTCAGA	ACAGCCCAGC	GTGATCCTTT
					SacIII		
	841	TAATGTGCTA	GTCTACGTGC	CCACTGGGTT	TGCTGTGTTT	GTGCCGACTG	AGCGAGATTT
	901	TCAGAGGAGG	GATCTGGTCC	GTTTCCAGAC	CTGCTGCTTC	CGGCATCA	

25 The Inverted Terminal Repeat (ITR) is shown in bold. Enzyme sites of interest are underlined with the enzyme name below. Putative TATA for E4 region is also shown.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine adenovirus genome.

2. A recombinant vector including a recombinant porcine adenovirus stably incorporating, and capable of expressing DNA of interest.

3. A recombinant vector as claimed in claim 2 wherein said recombinant porcine adenovirus is capable of expression of at least one heterologous nucleotide sequence.

4. A recombinant vector as claimed in claims 2 or 3 wherein said recombinant porcine adenovirus includes a live porcine adenovirus having virion structural proteins unchanged from those in a native porcine adenovirus from which said recombinant porcine adenovirus is derived.

5. A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.

6. A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.

7. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes antigenic determinants of infectious agents causing intestinal diseases in pigs.

8. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes antigenic determinants of infectious agents causing respiratory diseases in pigs.

9. A recombinant vector as claimed in claim 5 where said heterologous sequence encodes an antigenic determinant of pseudorabies virus (Aujeszky's disease virus).

10. A recombinant vector as claimed in claim 9 where heterologous sequence encodes an antigenic determinant of glycoprotein D of pseudorabies virus.

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11. A recombinant vector as claimed in claim 5 where said heretologous sequence encodes an antigenic determinant of porcine respiratory and reproductive syndrome virus (PRRSV).

12. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of Hog cholera virus.

13. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine parvovirus.

14. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine coronavirus.

15. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine rotavirus.

16. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine parainfluenza virus.

17. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of Mycoplasma hyopneumonia.

18. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes FLT-3 ligand.

19. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes interleukin 3 (IL-3).

20. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine interleukin 4 (IL4).

21. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes gamma interferon (γIFN).

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22. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte macrophage colony stimulating factor (GM-CSF).

23. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte colony stimulating factor (G-CSF).

24. A recombinant vector as claimed in claims 3 or 4 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.

25. A recombinant vector as claimed in any one of claims 2 to 24 wherein said recombinant porcine adenovirus is selected from the group consisting of serotypes 3 and 4.

26. A recombinant vector as claimed in any one of claims 2 to 25 wherein DNA of interest is stably integrated into the non-essential regions of the porcine adenovirus genome.

27. A recombinant vector as claimed in any one of claims 2 to 26 wherein DNA of interest is stably integrated into the right hand end of the genome.

28. A recombinant vector as claimed in claim 27 wherein DNA of interest is stably integrated into the right hand end of the genome at map units 97 to 99.5.

29. A recombinant vector as claimed in any one of claims 2 to 26 wherein DNA of interest is stably integrated into the E3 region of the genome.

30. A recombinant vector as claimed in claim 29 wherein DNA of interest is stably integrated into the E3 region of the genome at map units 81-84.

31. A method of producing a recombinant porcine adenovirus vector for use as a vaccine including inserting into a non-essential region of an porcine adenovirus genome, at least one heterologous nucleotide sequence in association with an effective promoter sequence.

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32. A method as claimed in claim 31 wherein prior to insertion of said heterologous nucleotide sequence, a restriction enzyme site is inserted into said non-essential region of said porcine adenovirus genome.

33. A recombinant vaccine for generating and/or optimising antibodies or cell mediated immunity so as to provide or enhance protection against infection by an infectious organism in pigs, said vaccine including at least one recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence, and suitable carriers and/or excipients.

34. A recombinant vaccine as claimed in claim 33 wherein the said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.

35. A recombinant vaccine as claimed in claim 33 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.

36. A recombinant vaccine as claimed in claim 33 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.

37. A recombinant vaccine as claimed in any one of claims 33 to 36 wherein said carriers and/or excipients are selected such that said vaccine is deliverable in the form of an aerosol spray, an enteric coated dosage unit or an inoculum.

38. A method of producing a recombinant vaccine as claimed in any one of claims 33 to 36 including admixing at least one recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence together with suitable carriers and/or excipients.

39. A method of vaccination of pigs against disease including administering to said pigs a first recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence encoding an antigenic determinant of said disease against which vaccination is desired.

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40. A method as claimed in claim 39 including administering to said pig a second porcine adenovirus vector including at least one heterologous nucleotide sequence which differs from said at least one heterologous nucleo-tide sequence incorporated in said first recombinant porcine adenovirus vector.

41. A method as claimed in claim 40 wherein said second porcine adenovirus vector comprises a serotype different to that of said first porcine adenovirus vector.

42. A method as claimed in claim 39 wherein said second porcine adenovirus vector incorporates, and is capable of expression of at least one heterologous nucleotide sequence encoding an immuno-potentiating molecule.

DATED this 11th day of November 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00648

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 039/235 ; C12N 15/63, 15/67. 15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Derwent Database - WPAT ; Chemical Abstracts - Keywords below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Derwent Database - JAPIO, USPM; Medline - Keywords below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, USPM: Keywords- (swine# or porcine# or hog# or pig#) (20N) adenovir:
Chemical Abstracts, Medline: Keywords- adenovir? (20N) (swine or porcine or hog or pig)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Derwent Abstract Accession Number: 97-310593 (WO 97/20036 (CYANAMID IBERICA) publ. 5 June 1997) (See examples)	1-4, 25-27, 31-33, 39 5-24, 34-38, 40-42
Y	AU, A, 72646/94 (RHONE POULENC RORER) OPI. 13 February 1995 (see page 2, Examples, Claims 9 and 10)	1-27, 31-42
X Y	Advances in Experimental Medicine and Biology, Vol. 342, 1993, P. Callebaut et al. "Construction of a Recombinant Adenovirus for the Expression of the Glycoprotein S Antigen of Porcine Respiratory Coronaviruses" pp 469- 470 (see entire document)	1-4, 25-27, 31-33, 39 5-24, 34-38, 40-42

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

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Date of the actual completion of the international search	Date of mailing of the international search report
8 October 1998	16 OCT 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer KAREN TAN Telephone No.: (02) 6283

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00648

C (Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Arch. Virology, Vol. 133, 1993, S.B Kleiboeker et al. "Genomic Cloning and Restriction Site Mapping of a Porcine Adenovirus Isolate:Demonstration of Genetic Stability in Porcine Adenovirus" pp 357-368 (see entire document) [cited in application]	1-4, 25-27, 31-33, 39 5-24, 34-38, 40-42
X Y	J. of Virology, Vol. 66, No. 7, July 1992, M. Gorziglia and A.Z Kapikian, "Expression of the OSU Rotavirus Outer Capsid Protein VP4 by an Adenovirus Recombinant" pp 4407-4412 (see entire document)	1-4, 25-27, 31-33, 39 5-24, 28, 34-38, 40-42
X Y	Virus Research, Vol. 31, 1994, S.B Kleiboeker, "Sequence Analysis of Putative E3, pVIII, and Fiber Genomic Regions of a Porcine Adenovirus" pp 17-25 (see entire document) [cited in application]	1-4, 25-27, 29-33, 39 5-24, 28, 34-38, 40-42
X Y	Virus Research, Vol. 36, 1995, P.S. Reddy et al., "Sequence Analysis of Putative pVIII, E3 and Fiber Regions of Porcine Adenovirus Type 3" pp 97-106 (see entire document)	1-4, 25-27, 29-33, 39 5-24, 28, 34-38, 40-42
X Y	Virus Research, Vol. 43, 1996, P.S. Reddy et al., "Porcine Adenoviruses Types 1, 2, and 3 have Short and Simple Early E3 Regions" pp. 99-109 (see entire document)	1-4, 25-27, 29-33, 39 5-24, 28, 34-38, 40-42
X Y	J. of Virology, Vol. 70, No. 6, June 1996, J.M. Torres et al., "Tropism of human adenovirus Type 5-based vectors in swine and their ability of protect against transmissible gastroenteritis coronavirus" pp. 3770-3780 (see entire document)	1-4, 25-27, 29-33, 39 5-24, 28, 34-38, 40-42
A	Intervirology, Vol. 36, 1993, P.S. Reddy et al., "Restriction Endonuclease Analysis and Molecular Cloning of Porcine Adenovirus Type 3" pp. 161-168 (see entire document) [cited in application]	1-42

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
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Patent Document Cited in Search Report		Patent Family Member					
WO	97/20036	AU	71325/96	ES	1032067	ES	2105984
AU	72646/94	WO	95/02697	EP	667912	FI	951138
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		CA	2144040	CN	1113390	CZ	9500639
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END OF ANNEX

CORRECTED VERSION*

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicants *(for all designated States except US)*: COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). PIG RESEARCH DEVELOPMENT CORPORATION [AU/AU]; Computer Associate House, 10 National Circuit, Barton, ACT 2600 (AU).

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Published
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(54) Title: RECOMBINANT PORCINE ADENOVIRUS VECTOR

(57) Abstract

This invention relates to a recombinant vector including a recombinant porcine adenovirus, stably incorporating and capable of expression of at least one heterologous nucleotide sequence. The nucleotide sequence is preferably one which encodes an antigenic determinant of Hog Cholera Virus or Pseudorabies virus. The further invention relates to a method of production of recombinant vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to methods of protecting pigs from disease.

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RECOMBINANT PORCINE ADENOVIRUS VECTOR

FIELD OF INVENTION

This invention relates to delivery vectors for antigen producing genes (heterologous gene sequences or fragments thereof) used to generate immune responses in commercial pigs susceptible to decimation by disease. Such vectors are especially useful for the preparation of vaccines which can be easily administered on a large scale to protect pigs against disease. This invention also relates to a method of production of suitable delivery vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to a method protecting pigs from disease.

BACKGROUND

The productivity of the intensive pig industry depends on the control of infectious diseases. Whilst diseases can be controlled in part by good hygiene and quarantine measures, the industry must still rely on vaccination to protect herds. In a commercial situation, the cost per animal is high in terms of feed and current disease control costs and therefore, the costs in disease prevention and control by any newly proposed vaccine must be cheap, effective and easy to deliver.

Conventionally, vaccines constituting live viral particles have been prepared by virus passage and selection of attenuated forms. Alternatively, killed vaccines were prepared from virulent viruses.

The most recent description of the use of viral vectors in the control of disease in pigs was the deletion mutant of pseudorabies virus for the control of Aujesky's disease. The use of a herpesvirus as a vector has the advantage of being able to stimulate a humoral and cell-mediated response, thus providing possible life long protection. Another advantage is the ability to insert other heterologous sequences in this vector, being expressed from a suitable promoter, to produce antigens for exposure to the animals immune system, thus protecting against two diseases. There are disadvantages of this system. Firstly, there is the issue of latency. Herpesviruses have the ability to intergrate into the neurons in ganglia for the life of the animal. It only requires a suitable stress on the animal to cause the reactivation of the virus and consequently full

disease. However, it is now known that the deletion of a specific gene, glycoprotein E, will attenuate the virus and prevent reactivation from latency. Therefore, this deletion vector is now widely used as an eradication vector for Aujesky's disease and subsequently will not be available as a suitable vector for the delivery of other antigens.

It is thus the aim of this invention to provide a delivery vehicle for heterologous sequences of genetic material that is particularly suited to administration on a large scale.

In particular, it is the aim of this invention to provide or enhance means for generation and/or optimisation of antibodies or cell-mediated immunity so as to provide protection against infection with common porcine diseases. It is an additional aim to provide a process for preparation of a suitable means for generation and/or optimisation of antibodies or cell-mediated immunity so as to protect pigs against infection with common porcine diseases. It is a further aim to provide a protection strategy.

SUMMARY OF INVENTION

The invention provides, in one embodiment, a recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine adenovirus genome.

In another embodiment the invention provides a recombinant vector including a recombinant porcine adenovirus which stably incorporates at least one heterologous nucleotide sequence. Preferably the heterologous nucleotide sequence is capable of expression as an antigenic polypeptide. The antigenic polypeptide encoded by at least one nucleotide sequence is preferably foreign to the host vector.

In a further embodiment of the present invention the heterologous nucleotide sequence is capable of expression as an immuno-potentiator molecule.

It is also to be understood that the heterologous nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

The recombinant vector may comprise a live recombinant porcine adenovirus in which the virion structural proteins are unchanged from those in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

This invention is partially predicated on the discovery that there are non-essential regions in the porcine adenovirus genome which do not correspond to those characterised previously on other adenoviruses thus making this virus particularly suited to delivery of heterologous sequences.

This invention is also predicated on the discovery that the porcine adenovirus generates a prolonged response in pigs thus making it well suited as a vaccine vehicle. Furthermore, the existence of a number of serotypes specific to respiratory or gastrointestinal tracts, allows the selection of a vaccine vehicle suited to a target organ and the type of immune response required.

The invention is also predicated on the discovery that porcine adenovirus can package genomic DNA greater than the 105% rule for mammalian adenoviruses with intermediate size genomes and that the resultant packaged virions are stable in vitro and in vivo.

Adenoviruses are a large and diverse family, having been isolated from many living species, including man and other mammals as well as a variety of birds. As a result adenoviruses have been separated into at least two genera, the *Mastoadenoviridae* and the *Aviadenoviridae*, and more recently a third genera has been proposed, the *Atadenovirdae*, which includes some bovine and avian adenoviruses (egg drop syndrome) (Benkö and Harrach, Archives of Virology 143, 829-837, 1998).

Porcine adenoviruses are prevalent infectious agents of pigs and to date four distinct serotypes have been recognised (Adair and McFerran, 1976) and evidence for at least one more (Derbyshire *et al.*, 1975). Of the four serotypes found, three (serotypes 1 to 3) were isolated from the gastrointestinal tract while the fourth was recovered from the respiratory system. The porcine adenoviruses are considered to be a low pathogenic widespread agent and although isolations were made in general from diseased animals, it was most likely that the adenovirus was present only as a secondary infection. They have been

isolated from pigs with diarrhoea and respiratory infections but it has been considered that at least the gastrointestinal adenovirus infections are usually asymptomatic (Sanford and Hoover, 1983). Porcine adenoviruses are spread by ingestion or inhalation and experimental infection via oral, intranasal and intratracheal inoculations have resulted in uptake of the virus. Experimental pathogenicity studies have shown that the primary sites of infection are the lower small intestine probably the tonsil (Sharpe and Jessett, 1967; Shadduck *et al.*, 1968). With serotype 4 infection, a viraemia appears to develop in experimental infections. However, this may be a less common manifestation with the gastrointestinal serotypes (Shadduck *et al.*, 1968). Faecal excretion is the most common cause for spread of PAV, being present for several weeks post infection. Nasal shedding also occurs under experimental conditions. PAV's role in pneumonia has been suggested to be that of either a predisposing factor or a synergist (Kasza *et al.*, 1969; Schiefer *et al.*, 1974) but experimental pneumonia with serotype 4 did not require a second agent to produce disease (Smith *et al.*,1973).

Porcine adenoviruses have yet to be examined in much detail and little is known about their role in disease or how common they are. This is due to the fact that they do not produce any significant disease in herds and have failed to draw the interest of industry through loss of production. It is likely that the number of serotypes of porcine adenoviruses is much greater than four and that it probably exists in the majority of pig herds as a normal commensal.

Work done on porcine adenovirus in regards to its morphology and molecular biology, has shown some similarities with other *Mastadenoviruses* examined. Its morphology is that of other adenoviruses examined with an icosahedral capsid containing a core of a double stranded DNA genome. Very little work on the characterisation of the PAV genome has been published (Benkö *et al.*, 1990, Kleiboeker *et al.*, 1993, Reddy *et al.*, 1993, Kleiboeker, 1994). The size of the PAV genome (approx. 34.8 kb) is slightly smaller than that of human adenoviruses (approx. 35.9 kb). One study has shown using hybridisation with DNA probes from the total genome of human adenovirus type 2 that there is reasonable DNA homology between the porcine and human

adenoviruses (Benkö *et al.*, 1990). A recent report on the serotype 4 PAV demonstrated that its genomic layout was also similar to that of the human adenoviruses in the area of the L4 and E3 regions (including the 33K and pVIII genes) even though the sequence homology was not as strong as may have been expected (Kleiboeker, 1994).

While choosing appropriate PAV for development as a live vectors to deliver vaccines to pigs, it is important to take into account the natural prevalence of serotypes. Those serotypes not commonly encountered in the field have an obvious advantages over those to which pigs are frequently exposed and to which they may have developed immunity.

A further consideration is the ability of the vector to remain active in the pig beyond the period which maternal antibodies in colostrum protect pigs immediately post-birth.

Other important considerations in choosing potential PAV vectors are pathogenicity and immunogenicity. Preferably live vector viruses should be highly infectious but non-pathogenic (or at least attenuated) such that they do not themselves adversely affect the target species.

The preferred candidates for vaccine vectors are non-pathogenic isolates of serotype 4 (respiratory) and serotype 3 (gastrointestinal). Serotype 3 has been chosen as the serotype of choice due to excellent growth abilities in continuous pig kidney cell lines. The isolation of other serotypes, which seems likely, may well alter this selection. It is notable that the more virulent strains produce a greater antibody response.

Heterologous nucleotide sequences which may be incorporated into non-essential regions of the viral genome and which may encode the antigenic determinants of infectious organisms against which the generation of antibodies or cell-mediated immunity is desirable may be those expressing antigenic determinants of intestinal infections caused by gastrointestinal viruses; for example rotavirus or parvovirus infections, or respiratory viruses, for example parainfluenza virus, or that of Japanese encephalitis.

Heterologous nucleotide sequences which may be incorporated include the antigenic determinants of the agents of:

Porcine parvovirus

Mycoplasma hyopneumonia

Porcine parainfluenza

Transmissable gastroenteritis (porcine coronavirus)

Porcine rotavirus

Hog cholera virus (Classical swine fever)

Swine dysentery

African swine fever virus

Pseudorabies virus (Aujesky's disease virus), in particular the glycoprotein D of the pseudorabies virus

Porcine respiratory and reproductive syndrome virus (PRRSV)

Heterologous nucleotide sequences more preferred for incorporation in the vectors of the invention are those expressing antigenic determinants of porcine parvovirus, porcine rotavirus, porcine coronavirus and classical swine fever virus.

It is also envisaged the heterologous sequences incorporated may be immuno-potentiator molecules such as cytokines or growth promoters, for example porcine interleukin 4 (IL4), gamma interferon (γIFN), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), FLT-3 ligand and interleukin 3 (IL-3).

The type of immune response stimulated by candidate vectors may affect the selection of heterologous nucleotide sequences for insertion therein. PAV serotypes 1, 2 and 3, which naturally infect via the gut may induce local mucosal immunity and are thus more suitable for infections of the intestines (eg classical swine fever virus). PAV serotype 4, which naturally infects via the respiratory system, may be more suitable for infections of the respiratory tract (eg porcine parainfluenza) may also induce good local immunity.

The DNA of interest which may comprise heterologous genes coding for antigenic determinants or immuno-potentiator molecules may be located in at least one non-essential region of the viral genome.

Non-essential regions of the viral genome which may be suitable for the purposes of replacement with or insertion of heterologous nucleotide

sequences may be non-coding regions at the right terminal end of the genome at map units 97 to 99.5. Preferred non-coding regions include the early region (E3) of the PAV genome at map units 81-84.

The heterologous gene sequences may be associated with a promoter and leader sequence in order that the nucleotide sequence may be expressed in situ as efficiently as possible. Preferably the heterologous gene sequence is associated with the porcine adenoviral major late promoter and splice leader sequence. The mammalian adenovirus major late promoter lies near 16-17 map units on the adenovirus genetic map and contains a classical TATA sequence motif (Johnson, D.C., Ghosh-Chondhury, G., Smiley, J.R., Fallis, L. and Graham, F.L. (1988), Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology 164, 1-14).

The splice leader sequence of the porcine adenovirus serotype under consideration is a tripartite sequence spliced to the 5' end of the mRNA of all late genes.

The heterologous gene sequence may also be associated with a poly adenylation sequence.

Instead of the porcine adenoviral major late promoter, any other suitable eukaryotic promoter may be used. For example, those of SV40 virus, cytomegalovirus (CMV) or human adenovirus may be used.

Processing and poly adenylation signals other than those of porcine adenoviruses may also be considered, for example, that of SV40.

In a further aspect of the invention there is provided a recombinant vaccine for generating and/or optimising antibodies or cell-mediated immunity so as to provide or enhance protection against infection with an infectious organism in pigs, the vaccine including at least one recombinant porcine adenovirus vector stably incorporating at least one heterologous nucleotide sequence formulated with suitable carriers and excipients. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide or as an immuno-potentiator molecule. More preferably, the heterologous nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

The antigenic polypeptide encoded by the at least one nucleotide sequence is preferably foreign to the host vector. At least one nucleotide sequence may be associated with a promoter/leader and a poly A sequence.

The recombinant vaccine may include live recombinant porcine adenovirus vector in which the virion structural proteins are unchanged from that in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

Preferred vector candidates for use in the recombinant vaccine are PAV isolates of serotype 3 and 4. Use of other serotypes is possible, depending on herd existing immunity and its environment.

The vaccine may be directed against respiratory and intestinal infections caused by a variety of agents. In order to direct the vaccine against a specific infectious organism, heterologous gene sequences encoding the antigenic determinants of those infectious organisms may be incorporated into non-essential regions of the genome of the porcine adenovirus comprising the vector. If the vaccine is to be used to optimise protection against disease, suitable heterologous nucleotide sequences may be those of immuno-potentiators such as cytokines or growth promoters.

The vaccine may comprise other constituents, such as stabilisers, excipients, other pharmaceutically acceptable compounds or any other antigen or part thereof. The vaccine may be in the form of a lyophilised preparation or as a suspension, all of which are common in the field of vaccine production.

A suitable carrier for such as a vaccine may be isotonic buffered saline.

In a further aspect of the invention, there is provided a method of preparing a vaccine for generation and/or optimisation of antibodies or cell-mediated immunity so as to induce or enhance protection against an infectious organism in a pig, which includes constructing a recombinant porcine adenovirus vector stably incorporating at least one heterologous nucleotide sequence, and placing said recombinant porcine adenovirus vector in a form suitable for administration. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide although it may also be an immuno-potentiator molecule. More preferably, the nucleotide sequence may encode

for and/or express, an antigenic polypeptide and an immuno-potentiatormolecule. The nucleotide sequence is conveniently foreign to the host vector.

Even more preferably the nucleotide sequence is associated with promoter/leader and poly A sequences.

The form of administration may be that of an enteric coated dosage unit, an inoculum for intra-peritoneal, intramuscular or subcutaneous administration, an aerosol spray, by oral or intranasal application. Administration in the drinking water or in feed pellets is also possible.

In another aspect of the invention, there is provided a method of producing a porcine adenovirus vaccine vector which includes inserting into a porcine adenovirus at least one heterologous nucleotide sequence. Said heterologous nucleotide sequence is preferably capable of expression as an antigenic polypeptide although it may also be an immuno-potentiator molecule. More preferably, the nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

Preferably the antigenic polypeptide encoded by the at least one nucleotide sequence is foreign to the host vector.

More preferably the heterologous nucleotide sequence is associated with promoter/leader and poly A sequences.

In one method of construction of a suitable vector the non-essential region to be altered to incorporate foreign DNA could be constructed via homologous recombination. By this method the non-essential region is cloned and foreign DNA together with promoter, leader and poly adenylation sequences is inserted preferably by homologous recombination between flanking sequences. By this method also, deletion of portions of the non-essential region is possible to create extra room for larger DNA inserts that are beyond the normal packing constraints of the virus.

By this method a DNA expression cassette containing an appropriate PAV promoter with foreign gene sequence as well as leader sequences and poly adenylation recognition sequences can be constructed with the unique restriction enzyme sites flanking the cassette enabling easy insertion into the

PAV genome.

In another aspect of the invention there is provided strategies for administration of the vaccines of the invention.

In one strategy, a heterologous antigen and immuno-modulatory molecule such as a cytokine may be expressed in the same recombinant and delivered as a single vaccine.

In one strategy according to the invention PAV vector based vaccines may be administered as 'cocktails' comprising 2 or more virus vectors carrying different foreign genes or immuno-potentiators.

In a preferred vaccination strategy of the invention, the 'cocktail' or simultaneous strategy, a vaccine based on both PAV serotype 3 and serotype 4 is used.

In another preferred strategy, a base recombinant serotype 3 porcine adenovirus is constructed and the fiber gene from serotype 4 replacing that of serotype 3 or the fiber from serotype 4 additionally cloned into the vaccine to broaden the targeting of the invention to both gut and respiratory delivery.

In an alternative strategy according to the invention, PAV vector based vaccines may be administered consecutively of each other to either administer booster vaccines or new vaccines at some stage subsequent to initial PAV vaccination. The vaccines used are preferably based on heterologous PAV isolates.

In a preferred version of the "consecutive" strategy, vaccines based on isolates serotypically unrelated are selected so as to achieve maximum protection against infection. In one example of such a strategy a vaccine based on PAV serotype 3 is administered subsequently or prior to vaccination with a vaccine based on PAV serotype 4.

Pigs are conveniently inoculated with vector vaccines according to the invention at any age. Piglets may be vaccinated at 1 day old, breeders may be vaccinated regularly up to point of giving birth and thereafter.

Preferably according to either the consecutive strategy or the cocktail strategy, pigs are vaccinated while still not fully immunocompetent. More conveniently, day-old pigs can be vaccinated for protection against re-infection

after a period of 4 weeks subsequent to initial vaccination.

In a further embodiment of the invention there is provided a method for producing an immune response in a pig including administering to the pig an effective amount of a recombinant vaccine according to the invention. An effective amount is an amount sufficient to elicit an immune response, preferably at least 10^4 $TCID_{50}$ per dose.

The vaccine of the invention may of course be combined with vaccines against other viruses or organisms such as parvovirus or Aujeszky's disease at the time of its administration.

In a preferred aspect of this embodiment of the invention, administration is by oral delivery or intra-nasally.

Methods for construction and testing of recombinant vectors and vaccines according to this invention will be well known to those skilled in the art. Standard procedures for endonuclease digestion, ligation and electrophoresis were carried out in accordance with the manufacturer's or suppliers instructions. Standard techniques are not described in detail and will be well understood by persons skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the DNA restriction endonuclease map of the entire PAV serotype 3 genome.

Figure 2 illustrates the sequence characterisation and cloning of the major later promoter and splice leader sequences of PAV serotype 3.

Figure 3 illustrates the sequences of the major later promoter, upstream enhancer sequence and splice leaders 1, 2 and 3.

Figure 4 illustrates the terminal 720 bases of the right end of the genome.

Figure 5 illustrates the promoter region of E3 and the overlapping L4 area.

Figure 6 illustrates a preferred method of construction of a PAV vector.

Figure 7 represents temperature data of pigs vaccinated with a PAV based vaccine following challenge with CSFV antigen.

Figure 8 graphically represents anti-PAV antibody levels detected by ELISA in pigs pre and post vaccination with a PAV based vaccine.

Figure 9 graphically illustrates the development of neutralising antibodies in pigs vaccinated with a PAV based vaccine pre and post challenge with CSFV antigen.

Figure 10 graphically illustrates the mean white blood cell (WBC) counts of pigs vaccinated with a recombinant PAV vaccine expressing porcine G-CSF.

Figure 11 graphically illustrates the percentage change in white blood cell (WBC) counts following vaccination with a recombinant PAV vaccine expressing porcine G-CSF.

Figure 12 graphically represents the percentage change in monocyte cell populations following vaccination with recombinant PAV-G-CSF.

Figure 13 graphically represents the percentage change in lymphocyte cell populations following vaccination with recombinant PAV-G-CSF.

Figure 14 graphically represents the change in stimulation of T-cells following vaccination with recombinant PAV-G-CSF.

PREFERRED EMBODIMENTS

Aspects of preferred embodiments of the invention based on PAV isolates serotype 3 and serotype 4 will now be described. Whilst these two isolates have been selected because of their sites of infection in the pig, it will be appreciated that other isolates of porcine adenovirus may be more suitable for construction of vaccine vectors provided the criteria for selection described herein before are met.

In general, PAV are considered of low pathogenicity with little consequence in the field. The pathogenic significance of PAV is reviewed in Derbyshire, 1989. The first report of isolation of PAV was from a 12 day old pig with diarrhoea (Haig *et al.*, 1964). Two years later, PAV type 4 was first reported, isolated from the brain of a pig suffering from encephalitis of unknown cause (Kasza, 1966). Later reports have associated PAV mainly with diarrhoea in the field although this is normally low grade. PAV can also be regularly isolated from healthy animals with no disease signs and it is quite likely that its isolation from diseased animals is more a coincidence of its prevalence than an indicator of pathogenicity. However, an association between serotype 4 and respiratory disease has been reported (Watt, 1978) and this has been supported

by experimental infection (Edington *et al.*, 1972). Experimental infections with gastrointestinal serotypes of the virus (eg serotype 3) have been able to produce diarrhoea but the pathological changes produced were not clinically significant.

The genome of the selected PAV serotype 3 was characterised by conventional methods. The DNA restriction endonuclease maps of the entire genome is illustrated in figure 1. The genomes are orientated left to right. By convention adenovirus genomes are normally orientated such that the terminal region from which no late mRNA transcripts are synthesised is located at the left end. The enzymes used to generate the map are indicated at the edge of each map.

CHARACTERISATION OF MAJOR LATE PROMOTER (MLP) AND SPLICE LEADER SEQUENCES (LS) OF PAV SEROTYPE 3

Identification and cloning of the PAV MLP

By use of restriction enzyme and genetic maps of the PAV serotype 3 genome, a region was located that contained the MLP and leader sequences (Fig 1). The fragments identified in this region were cloned into plasmid vectors and sequenced.

The MLP promoter sequence was identified as containing a classical TATA sequence, the only one in the region sequenced, as well as upstream factors and was subsequently confirmed by the location of the leader sequence and the transcriptional start site.

Figures 2 and 3 illustrate the sequence characterisation of the major late promoter and splice leader sequences of PAV serotype 3.

In order to determine the structure and sequence of the leader sequence spliced to late mRNA, porcine kidney cells were infected with PAV and the infection was allowed to proceed until late in the infection cycle (usually 20-24 hr p.i.). At this time total RNA was purified from the infected cells using the RNAgents total RNA purification kit (Promega). The isolated RNA was precipitated with isopropanol and stored at -70°C in 200 µl aliquots until

required. Poly A (mRNA) was isolated from total RNA by the use of the Poly AT tract System (Promega, USA). The isolated mRNA was used in cDNA production.

For cDNA production, oligonucleotides were produced to the complimentary strand of the hexon gene and the penton base gene, both being MLP transcripts. A further oligonucleotide was produced which covered the proposed cap site of the major late transcript, 24 bases downstream of the TATA box. This oligonucleotide was used in conjunction with that used in cDNA production in Taq polymerase chain reaction. The resulting DNA produced from positive clones was digested with appropriate restriction enzymes to determine the size of the inserted fragment. DNA sequencing of these inserted fragments was performed using a modification of the chain termination technique (Sanger, F., Nicklen, S and Gulson, A.R., 1977, DNA sequencing with chain terminating inhibitors. PNAS USA 74: 5463-5467) so as to allow Taq DNA polymerase extension (Promega, USA).

To confirm the leader sequence cap site, fresh cDNA was prepared and this time a tail of dGTP residues added to it. Briefly, cDNA was incubated with 1 mM dGTP and approximately 15 units of terminal deoxynucleotidyl transferase (Promega) in 2 mM CaCl2 buffer at 37°C for 60 minutes. The reaction was stopped by heating to 70°C for 10 minutes. The DNA was then ethanol precipitated and resuspended in a volume suitable for use in polymerase chain reaction (PCR). PCR was performed as previously described using a poly (dC) oligonucleotide with a *Xba*I site at the 5' end. Resulting fragments were blunt ended with T4 DNA polymerase at 37°C for 30 minutes in the presence of excess nucleotides and cloned into the *Sma*I site of the pUC18 vector. DNA preparation and sequencing were performed, as described previously, on clones shown to be positive by hybridisation.

Figure 3 illustrates the separate sequences of the major late promoter, upstream enhancer sequence and splice leaders 1, 2 and 3 as determined from cDNA studies. Figure 2 illustrates the DNA sequence of the complete promoter cassette with the components joined together.

CHARACTERISATION OF NON-ESSENTIAL REGIONS OF VIRAL GENOME

The right end was identified by cloning and complete sequencing of the PAV serotype 3 *Apa*I fragment J of approximately 1.8 Kbp. The inverted terminal repeat (ITR) has been determined by comparison of the RHE sequence with that of the left hand end. The ITR is 144 bases long and represents the starting point into which potential insertions can be made. Figure 4 shows the sequence of the terminal 720 bases. Restriction endonuclease sites of interest for insertion of foreign DNA are indicated in the terminal sequence. A putative TATA site for the E4 promoter is identified, this being the left most end for the possible site of insertion. Initial insertions will be made into the *Sma*I or *Eco*RI sites.

The E3 region of the genome, this also being a non-essential area, has been located and cloned. The promoter region of E3 has been identified and the overlapping L4 area sequenced (Figure 5). The region of the E3 after the polyadenylation signal of the L4 is also a possible site for insertion and can also be used for deletion to create more room for larger cassette insertions.

CONSTRUCTION OF PAV VECTOR

Figure 6 illustrates a preferred method of construction of a PAV vector. The right hand end *Apa*I fragment J of PAV serotype 3 is cloned and a unique *Sma*I restriction endonuclease site 230 bp from the inverted repeats was used as an insertion site.

The major late promoter expression cassette containing the E2 (gp55) gene of classical swine fever virus (hog cholera virus) was cloned into the *Sma*I site of the RHE fragment.

A preferred method of homologous recombination was cutting genomic PAV 3 DNA with *Hpa*I, a unique site in the genome, and transfecting this DNA with *Apa*I cut expression cassette plasmid containing gp55.

The DNA mix was transfected into preferably primary pig kidney cells by standard calcium chloride precipitation techniques.

The preferred method of transfection generates recombinant virus through homologous recombination between genomic PAV 3 and plasmid (Fig 6).

DETAILED DESCRIPTION OF THE INVENTION

CONSTRUCTION OF PAV VECTOR

The following examples show the construction of representative recombinant porcine adenoviruses of this invention. The recombinant viruses were propagated and titred on primary porcine kidney cells.

1 Construction of PAV-gp55

An expression cassette consisting of the porcine adenovirus major late promoter, the classical swine fever virus (CSFV) gene (gp55) and SV 40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.38 kilobase pairs. No deletion of the genomic PAV 3 was made. Mammalian adenoviruses with intermediate genomes (~36kb) have been shown to accommodate up to 105% of the wild-type genomic length, and genomes larger than this size are either unpackageable or extremely unstable, frequently undergoing DNA rearrangements (Betts, Prevec and Graham, Journal of Virology 67, 5911-5921 (1993), Packaging capacity and stability of human adenovirus type 5 vectors: Parks and Graham, Journal of Virology, 71, 3293-3298, (1997), A helper dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging). In this invention, PAV genomic length was 34.8 kb, into which was inserted without any other deletion an expression cassette of 2.38 kb. The resulting genomic DNA length of the recombinant porcine adenovirus of this invention was 106.8%, and therefore exceeded the putative maximum limit for construction of a stable recombinant. The recombinant virus was plaque purified three times and passaged stably in primary pig kidney cells. The recombinant was shown to contain gp55 by Southern blot hybridisation. Expression of gp55 was demonstrated by infecting primary PK cell line grown on glass cover slips with the recombinant porcine adenovirus. After 24 hours, immunoflouresencent staining (IF) showed infected cells expressing gp55.

2 Construction of recombinant PAV-G-CSF

An expression cassette comprising of the porcine adenovirus major late promoter, the gene encoding porcine granulocyte-colony stimulating factor (G-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 1.28 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and passaged stably in primary pig kidney cells. The recombinant was shown to contain G-CSF by Southern blot hybridisation and polymerase chain reaction (PCR). Expression of G-CSF was demonstrated by infecting primary kidney cells with the recombinant PAV-G-CSF. Tissue culture supernatants from the infected primary kidney cells were then electrophoresed in SDS-PAGE gels and transferred to filters. Infected cells expressing G-CSF were detected in a Western blot using a rabbit polyclonal antiserum against porcine G-CSF expressed by purified recombinant *E coli.*

3 Construction of recombinant PAV-gp55T/GM-CSF

An expression cassette consisting of the porcine adenovirus major late promoter, a truncated form of the classical swine fever virus gene gp55 fused in frame to the gene encoding either the full length or the mature form of porcine granulocyte/macrophage-colony stimulating factor (GM-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.1 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and shown to contain gp55 and GM-CSF by PCR.

4 Construction of recombinant PAV-gp55/E3

An expression cassette consisting of the porcine adenovirus major late promoter, the classical swine fever virus gene gp55 and SV40 polyA was inserted between the *BsrgI/SnaB*I sites in the E3 region (MU 81-84) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a

recombinant PAV 3. The size of the expression cassette was 2.38 kilobase pairs. A 620 base pair deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and shown to contain gp55 by PCR.

VACCINATION STRATEGY

1. Vaccination with PAV-gp55

In this experiment 5-6 week old piglets were used to represent immunocompetent pigs. A group of the piglets (#2, 6 and 7) were vaccinated with recombinant PAV-gp55 administered subcutaneously at a dose of 1 X 10^7 pfu per piglet. A control group of piglets (#3, 8, 11, 12, 13 and 14) were unvaccinated. No clinical signs were observed (no rise in temperature) in the vaccinated group of piglets (Table 1).

Table 1. Temperatures of pigs vaccinated with rPAV::gp55

Temperatures post vaccination with rPAV::gp55 CSFV (°C)								
Pig No.	Day 0	1	2	3	6	9	10	13
2	39.7	39.2	39.4	39.8	39.6	39.8	39.6	39.2
3 (control)	39.5	39.2	39.4	39.0	38.8	39.3	39.0	39.7
6	39.7	39.1	39.1	39.0	39.1	39.8	39.1	39.8
7	39.4	39.8	39.8	39.4	39.9	38.9	39.6	39.7
8 (control)	39.6	39.5	39.4	39.0	40.5	39.4	39.1	39.7

Five weeks after vaccination with the recombinant PAV-gp55 both groups of pigs were challenged with a lethal dose (1 X $10^{3.5}$ $TCID_{50}$) of virulent Hog Cholera virus (Classical swine fever virus) applied subcutaneously.

The temperatures of the pigs were monitored and the results tabulated in Table 2 and graphically represented in Figure 7.

Table 2 Temperatures post challenge with CSFV (°C)

Pig No.	Day -1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2		39.6	39.9	40.1	40.3	40.1	39.2	39.7	39.5	39.5	3	9					
7	39.4	39.1	39.5	39.1	40.0	39.4	38.9										
3		39.6	40.4	39.6	40.0	40.7	40.7	41.9	40.7	40.9	42.0+						
6		39.5	39.5	40.0	40.0	39.6	40.5	39.9	39.2	39.2	3	8					
8	39.3	38.8	38.9	39.6	39.3	39.1	38.9										
7		39.8	39.9	40.4	40.6	40.3	39.7	39.7	39.7	39.5	3	9					
3	39.1	39.3	39.6	40.6	39.7	39.8	39.7										
8		39.9	40.6	40.5	40.3	40.0	41.4	39.8	41.0	40.6	39.0+						
11		39.6	39.9	40.0	40.3	40.7	40.5	40.0	41.8	41.5	41.3+						
12		39.8	39.9	40.9	41.0	41.2	40.6	40.1	41.0	41.7	40.3+						
13		39.7	40.0	41.2	41.5	41.6	41.0	39.7+									
14		39.3	40.0	39.6	39.8	40.3	40.7	41.2	40.8	40.2	41.7+						

The results show that by day 5 the control group had elevated temperatures (greater than 40.5°C) and showed clinical signs of disease. The vaccinated group showed no clinical signs of disease. Pigs from the control group were dead or euthanased by day 9. The vaccinated group were euthanased at day 16. At post mortem all control pigs showed severe clinical disease, the vaccinated pigs showed no clinical signs of disease.

The results indicate that the pigs vaccinated subcutaneously with the recombinant PAV-gp55 survived challenge with classical swine fever virus at a lethal dose.

Sera were collected from both groups of pigs and tested for the presence of antibodies to PAV by ELISA. These tests showed the presence of pre-existing antibodies to PAV before vaccination. The level of these antibodies increased following vaccination with the recombinant PAV-gp55 to peak between days 28 and 36 post vaccination. These results are tabulated in Figure 8.

Sera were collected from the vaccinated group of pigs pre and post challenge with CSFV and tested in the presence of neutralising antibodies to CSFV. Sera were tested at days 0 and 28 after vaccination with recombinant PAV-gp55 (pre challenge) and then again at day 16 post challenge (day 52 after vaccination). The results in Figure 9 show no neutralising antibodies detected at day 0, low levels of neutralising antibodies at day 28 and high levels at day 52.

These results show that the recombinant PAV-gp55 can protect pigs from lethal challenge with classical swine fever virus in the presence of pre-existing antibodies to PAV.

2. Vaccination with PAV-G-CSF

In this experiment 5-6 week old piglets were used to represent immunocompetent pigs. A group of pigs (n=4) were vaccinated with recombinant PAV-G-CSF administered subcutaneously at a dose of 1 X 10^7 pfu per piglet. A second group (n=4) were vaccinated with PAV wild type (wt) administered subcutaneously at a dose of 1 X 10^7 pfu per piglet. A control group (n=4) were unvaccinated. Pigs were bled at 8 hour intervals for a period of 104 hours post vaccination. Complete blood counts were determined and the

mean white blood cell (WBC) counts for each group monitored. These results are graphically represented in Figure 10 and the percentage change in mean WBC counts graphically represented in Figure 11.

Pigs vaccinated with either PAV wt or PAV-G-CSF showed clinical signs of disease with mild diahorrea 24-72 hours post vaccination. Both groups of pigs were completely recovered by 80-96 hours post-vaccination. Control pigs showed no clinical signs of disease.

Complete blood screening results show that the mean WBC counts for control pigs increased over the duration of the experiment.

PAV wt vaccinated pigs also show an increase in WBC counts, with a depression in WBC counts between 48-80 hours post-vaccination and recovery from 80-96 hours onwards.

Pigs vaccinated with the recombinant PAV-G-CSF show a significant depression in WBC counts over the duration of the experiment. A statistical analysis of these results is tabulated in Table 3. The analysis shows that differences between the mean WBC counts (controls and PAV-G-CSF; PAV wt and PAV-G-CSF) were significant indicating that the recombinant PAV-G-CSF altered the proportions of cells involved with immunity.

Table 3. Results of t-tests between mean WBC counts of groups of pigs vaccinated with either PAV wild type (wt), PAV recombinant expressing G-CSF (PAV-G-CSF) or unvaccinated controls.

	Pre vacc O hr	8-24 hr[d]	32-48 hr	56-72 hr	80-104 hr
Control vs PAV-G-CSF[a]	p> 0.2[b]	p> 0.2	p> 0.2	p> 0.2	p< 0.005
Control vs PAV wt	p> 0.1	p> 0.01[c]	p> 0.02	p> 0.2	p< 0.05
PAV-G-CSF vs PAV wt	p> 0.2	p< 0.05	p< 0.05	p< 0.05	p< 0.001

a: null hypothesis; there is no difference between the mean WBC counts.

b: p>0.05, insufficient to reject the null hypotheses at the 95% confidence level, conclude that there is no difference between mean leucocyte levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean leucocyte levels.

d: 4 pigs in each group were bled at 8 hour intervals.

Differential WBC counts were also determined and monitored for each group. The percentage change in mean monocyte cell populations graphically represented in Figure 12 and the percentage change in mean lymphocyte cell populations graphically represented in Figure 13. Figure 12 shows that monocyte cell populations increased rapidly in pigs following vaccination with PAV wt, but were suppressed by vaccination with the recombinant PAV-G-CSF. This effect was due to the expression of G-CSF by the recombinant. A statistical analysis of these results is tabulated in Table 4. The analysis shows that there was a significant difference between the PAV wt and PAV-G-CSF from 32 to 96 hours post vaccination. Figure 13 shows that there were shifts in lymphocyte cell population numbers following vaccination with the recombinant PAV-G-CSF. Unvaccinated controls show stable lymphocyte cell numbers over the duration of the experiment, whereas pigs vaccinated with PAV wt show a significant increase in lymphocyte cell population as a response to infection. Pigs vaccinated with the recombinant PAV-G-CSF show a decline in lymphocyte cell population. A statistical analysis of these results is tabulated in Table 5. The analysis shows that there was a significant difference between PAV wt and the recombinant PAV-G-CSF between 8 and 96 hours post vaccination. The different responses in lymphocyte cell proliferation following vaccination with recombinant PAV-G-CSF and PAV wt were due to the expression of G-CSF by the recombinant. These results show that vaccination with recombinant PAV-G-CSF produces a shift in sub-populations of cells involved in immunity.

Table 4. Results of t-tests between mean monocyte cell populations following vaccination of pigs with either recombinant PAV-G-CSF, wild type PAV (PAV wt) or unvaccinated controls.

	pre vacc	8-24hr[d]	32-48hr	56-72hr	80-96hr	104hr
conrols vs PAV-G-CSF[a]	p>0.1[b]	p>0.2	p>0.2	p>0.2	p>0.2	p>0.2
controls vsPAV wt	p>0.2	p<0.002[c]	p>0.2	p<0.001[c]	p>0.2	p>0.2
PAV wt vsPAV-G-CSF	p>0.2	p<0.001[c]	p>0.2	p>0.2	p>0.1	p>0.05

a: null hypothesis; there is no difference between the mean monocyte cell counts.

b: p>0.1, insufficient to reject the null hypothesis at the 90% confidence level, conclude that there is no difference between mean monocyte cell levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean monocyte cell levels

d: 4 pigs in each group were bled at 8 hour intervals

Table 5. Results of t-tests between mean lymphocyte cell populations following vaccination of pigs with either recombinant PAV-G-CSF, wild type PAV (PAV wt) or unvaccinated controls.

	pre vacc	8-24hr[d]	32-48hr	56-72hr	80-96hr	104hr
conrols vs PAV-G-CSF[a]	p>0.2	p>0.05[b]	p>0.2	p>0.2	p>0.2	p>0.2
controls vsPAV wt	p>0.2	p>0.2	p<0.01[c]	p<0.001[c]	p<0.001[c]	p>0.2
PAV wt vs PAV-G-CSF	p>0.2	p<0.05[c]	p<0.002[c]	p<0.005[c]	p<0.001[c]	p>0.05

a: null hypothesis; there is no difference between the mean lymphocyte cell counts.

b: p>0.05, insufficient to reject the null hypothesis at the 95% confidence level, conclude that there is no difference between mean lymphocyte cell levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean lymphocyte cell levels.

d: 4 pigs in each group were bled at 8 hour intervals

Figure 14 graphically represents changes in the proliferation of T cells of each group following stimulation with Concanavalin A (Con A). These results confirm that there was a significant proliferation of T-cells following vaccination with PAV wt at day 2 post vaccination, whereas vaccination with the recombinant PAV-G-CSF resulted in a suppression of T-cell proliferation by day 3.

The results of vaccination with a recombinant PAV expressing porcine G–CSF shows that G-CSF has a significant effect on the cells involved with immune responses.

It will be appreciated that whilst this document establishes the metes and bounds of this invention, all embodiments falling within its scope for example with regard to heterologous genes, insertion sites, types of promoter and serotype have not necessarily been specifically exemplified although it is intended that they should fall within the scope of protection afforded this invention.

Figure 2

Total sequence of the PAV Major Late Promoter cassette including the added nucleotides 5' (upstream) of the USF.

Nucleotide base count: 76 A 143 C 187 G 96 T Total 502 bp

```
  1   GGTGCCGCGG TCGTCGGCGT  AGAGGATGAG  GGCCCAGTCG  GAGATGAAGG  CACGCGCCCA
 61   GGCGAGGACG AAGCTGGCGA  CCTGCGAGGG  GTAGCGGTCG  TTGGGCACTA  ATGGCGAGGC
121   CTGCTCGAGC GTGTGGAGAC  AGAGGTCCTC  GTCGTCCGCG  TCCAGGAAGT  GGATTGGTCG
181   CCAGTGGTAG TCCACGTGAC  CGGCTTGCGG  GTCGGGGGGT  ATAAAAGGCG  CGGGCCGGGG
241   TGCGTGGCCG TCAGTTGCTT  CGCAGGCCTC  GTCACCGGAG  TCCGCGTCTC  CGGCGTCTCG
301   CGCTGCGGCT GCATCTGTGG  TCCCGGAGTC  TTCAGGTCCT  TGTTGAGGAG  GTACTCCTGA
361   TCGCTGTCCC AGTACTTGGC  GTGTGGGAAG  CCGTCCTGAT  CGCGATCCTC  CTGCTGTTGC
421   AGCGCTTCGG CAAACACGCG  CACCTGCTCT  TCGGACCCGG  CGAAGCGTTC  GACGAAGGCG
481   TCTAGCCAGC AACAGTCGCA  AG
```

The Upstream Stimulatory Factor (USF) and TATA motiff are in **bold**. The complete leader sequence is italised with the cap site and splice sites between the individual leaders indicated by double underlining or single underlining respectively.

Figure 3

Individual sequences of the Promoter cassette components:

I. The 5' (upstream) sequence included in the long cassette.

```
  1    GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAGTCG GAGATGAAGG CACGCGCCCA
 61    GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121    CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181    CCAGTGGTAG
```

II. Sequence including the USF, TATA motiff and sequence to the cap site.

```
  1    CCACGTGACC GGCTTGCGGG TCGGGGGGTA TAAAAGGCGC GGGCCGGGGT GCGTGGCCGT
 61    C
```

III. First leader sequence.

```
  1    AGTTGCTTCG CAGGCCTCGT CACCGGAGTC CGCGTCTCCG GCGTCTCGCG CTGCGGCTGC
 61    ATCTGTGGTC CCGGAGTCTT CAG
```

IV. Second leader sequence.

```
  1    GTCCTTGTTG AGGAGGTACT CCTGATCGCT GTCCCAGTAC TTGGCGTGTG GGAAGCCGTC
 61    CTGATCG
```

V. Third leader sequence.

```
  1    CGATCCTCCT GCTGTTGCAG CGCTTCGGCA AACACGCGCA CCTGCTCTTC GGACCCGGCG
 61    AAGCGTTCGA CGAAGGCGTC TAGCCAGCAA CAGTCGCAAG
```

Figure 4

Sequence of the right hand end of the PAV genome this area being a proposed site for insertion of expression cassettes.

Nucleotide base count 183 A 255 C 306 G 204 T Total 948 bases

1	**CATCATCAAT**	**AATATACCGC**	**ACACTTTTAT**	**TGCCCCTTTT**	**GTGGCGTGGT**	**GATTGGCGGA**
61	**GAGGGTTGGG**	**GGCGGCGGGC**	**GGTGATTGGT**	**GGAGAGGGGT**	**GTGACGTAGC**	**GTGGGAACGT**
121	**GACGTCGCGT**	**GGGAAAATAA**	**CGTG**GCGTGG	GAACGGTCAA	AGTCCGAGGG	GCGGGGTCAA
181	AGTCCGCAGT	CGCGGGGCGG	AGCCGGCTGG	CGGGAATTCC (EcoRI)	CGGGACTTTC (SmaI)	TGGGCGGGTA
241	ATCGTTAACG (HpaI)	CGGAGGCGGG	GGAATTCCGA (EcoRI)	TCGGACGATG	TGGTACTGAT	TAACCGACCG
301	CAGGCGTGTC	CACATCCGCT	GTGGGTATAT	CACCGGCGCT	CGCGGTGTTC	GCTCACACTC
361	GTCTCGGCGC	TGTCACAGAG	AGAGACACTG	AGAGCGAGAC	GAGGAGAAAC	CGAAAGCGGG
421	GCAGGAGGAG	TCACCGGGCC	ATCTTCCCAT	CAGAGCCCTC	TCATGGCCCA	CGACCGACTG
481	CTGCTGGCCG	CGGTGGCTGA	CTGTTGCTCG	CCGTGCTCTA	TCTGTACTTC	GCCTACCTCG
541	CGTGGCAGGA	TCGGGACACT	CTTCACACTC	AGGAGGCCGC	CTCTCCTCGC	TTCTTCATCG
601	GGTCCAACCA	CCAGCCCTGG	TGCCCGGATT	TTGATTGGCA	GGAGCAGGAC	GAGCACACTC
661	ACTAGACGTT	TAGAAAAAAG	ACACACATTG	GAACTCA**TAT**	**ATGTCTGCGG**	GACCGCATCA
721	GCAGCCCGGT	CTGCTGTTGG	CTGCGGGTGA	GAGGCCTCCG (StuI)	GTAATTCATC	AGAACCGCAT
781	TCATCTGCGC	CACGTCCCGA	CATATGGTGC	TGACGTCAGA (SacIII)	ACAGCCCAGC	GTGATCCTTT
841	TAATGTGCTA	GTCTACGTGC	CCACTGGGTT	TGCTGTGTTT	GTGCCGACTG	AGCGAGATTT
901	TCAGAGGAGG	GATCTGGTCC	GTTTCCAGAC	CTGCTGCTTC	CGGCATCA	

The Inverted Terminal Repeat (ITR) is shown in bold. Enzyme sites of interest are underlined with the enzyme name below. Putative TATA for E4 region is also shown.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine adenovirus genome.

2. A recombinant vector including a recombinant porcine adenovirus stably incorporating, and capable of expressing DNA of interest.

3. A recombinant vector as claimed in claim 2 wherein said recombinant porcine adenovirus is capable of expression of at least one heterologous nucleotide sequence.

4. A recombinant vector as claimed in claims 2 or 3 wherein said recombinant porcine adenovirus includes a live porcine adenovirus having virion structural proteins unchanged from those in a native porcine adenovirus from which said recombinant porcine adenovirus is derived.

5. A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.

6 A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.

7 A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes antigenic determinants of infectious agents causing intestinal diseases in pigs.

8. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes antigenic determinants of infectious agents causing respiratory diseases in pigs.

9. A recombinant vector as claimed in claim 5 where said heterologous sequence encodes an antigenic determinant of pseudorabies virus (Aujeszky's disease virus).

10. A recombinant vector as claimed in claim 9 where heterologous sequence encodes an antigenic determinant of glycoprotein D of pseudorabies virus.

11. A recombinant vector as claimed in claim 5 where said heretologous sequence encodes an antigenic determinant of porcine respiratory and reproductive syndrome virus (PRRSV).

12. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of Hog cholera virus.

13. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine parvovirus.

14. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine coronavirus.

15. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine rotavirus.

16. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine parainfluenza virus.

17. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of Mycoplasma hyopneumonia.

18. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes FLT-3 ligand.

19. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes interleukin 3 (IL-3).

20. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine interleukin 4 (IL4).

21. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes gamma interferon (γIFN).

22. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte macrophage colony stimulating factor (GM-CSF).

23. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte colony stimulating factor (G-CSF).

24. A recombinant vector as claimed in claims 3 or 4 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.

25. A recombinant vector as claimed in any one of claims 2 to 24 wherein said recombinant porcine adenovirus is selected from the group consisting of serotypes 3 and 4.

26. A recombinant vector as claimed in any one of claims 2 to 25 wherein DNA of interest is stably integrated into the non-essential regions of the porcine adenovirus genome.

27. A recombinant vector as claimed in any one of claims 2 to 26 wherein DNA of interest is stably integrated into the right hand end of the genome.

28. A recombinant vector as claimed in claim 27 wherein DNA of interest is stably integrated into the right hand end of the genome at map units 97 to 99.5.

29. A recombinant vector as claimed in any one of claims 2 to 26 wherein DNA of interest is stably integrated into the E3 region of the genome.

30. A recombinant vector as claimed in claim 29 wherein DNA of interest is stably integrated into the E3 region of the genome at map units 81-84.

31. A method of producing a recombinant porcine adenovirus vector for use as a vaccine including inserting into a non-essential region of an porcine adenovirus genome, at least one heterologous nucleotide sequence in association with an effective promoter sequence.

32. A method as claimed in claim 31 wherein prior to insertion of said heterologous nucleotide sequence, a restriction enzyme site is inserted into said non-essential region of said porcine adenovirus genome.

33. A recombinant vaccine for generating and/or optimising antibodies or cell mediated immunity so as to provide or enhance protection against infection by an infectious organism in pigs, said vaccine including at least one recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence, and suitable carriers and/or excipients.

34. A recombinant vaccine as claimed in claim 33 wherein the said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.

35. A recombinant vaccine as claimed in claim 33 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.

36. A recombinant vaccine as claimed in claim 33 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.

37. A recombinant vaccine as claimed in any one of claims 33 to 36 wherein said carriers and/or excipients are selected such that said vaccine is deliverable in the form of an aerosol spray, an enteric coated dosage unit or an inoculum.

38. A method of producing a recombinant vaccine as claimed in any one of claims 33 to 36 including admixing at least one recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence together with suitable carriers and/or excipients.

39. A method of vaccination of pigs against disease including administering to said pigs a first recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence encoding an antigenic determinant of said disease against which vaccination is desired.

40. A method as claimed in claim 39 including administering to said pig a second porcine adenovirus vector including at least one heterologous nucleotide sequence which differs from said at least one heterologous nucleotide sequence incorporated in said first recombinant porcine adenovirus vector.

41. A method as claimed in claim 40 wherein said second porcine adenovirus vector comprises a serotype different to that of said first porcine adenovirus vector.

42. A method as claimed in claim 39 wherein said second porcine adenovirus vector incorporates, and is capable of expression of at least one heterologous nucleotide sequence encoding an immuno-potentiating molecule.

Fig 1.

Restriction enzyme maps of the PAV3 grnome

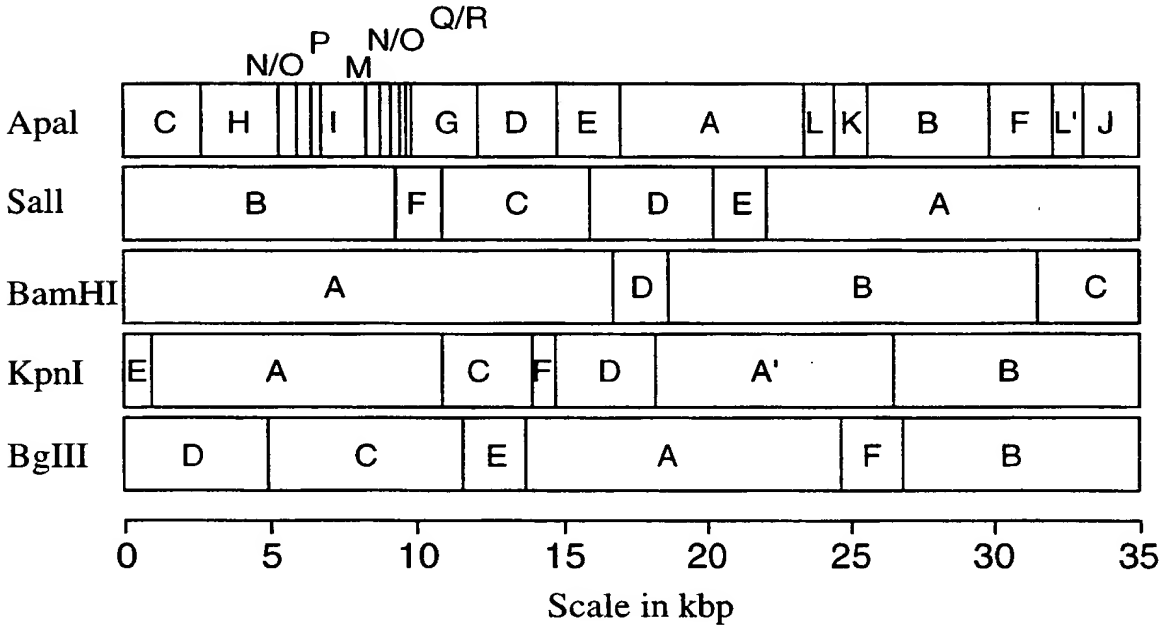


Fig 2.

Total sequence of the PAV Major Late Promoter cassette including the added nucleotides 5' (upstream) of the USF.

Nucleotide base count: 76 A 143 C 187 G 96 T Total 502 bp

```
  1   GGTGCCGCGG TCGTCGGCGT  AGAGGATGAG  GGCCCAGTCG  GAGATGAAGG  CACGCGCCCA
 61   GGCGAGGACG AAGCTGGCGA  CCTGCGAGGG  GTAGCGGTCG  TTGGGCACTA  ATGGCGAGGC
121   CTGCTCGAGC GTGTGGAGAC  AGAGGTCCTC  GTCGTCCGCG  TCCAGGAAGT  GGATTGGTCG
181   CCAGTGGTAG TCCACGTGAC  CGGCTTGCGG  GTCGGGGGGT  ATAAAAGGCG  CGGGCCGGGG
241   TGCGTGGCCG TCAGTTGCTT  CGCAGGCCTC  GTCACCGGAG  TCCGCGTCTC  CGGCGTCTCG
301   CGCTGCGGCT GCATCTGTGG  TCCCGGAGTC  TTCAGGTCCT  TGTTGAGGAG  GTACTCCTGA
361   TCGCTGTCCC AGTACTTGGC  GTGTGGGAAG  CCGTCCTGAT  CGCGATCCTC  CTGCTGTTGC
421   AGCGCTTCGG CAAACACGCG  CACCTGCTCT  TCGGACCCGG  CGAAGCGTTC  GACGAAGGCG
481   TCTAGCCAGC AACAGTCGCA  AG
```

The Upstream Stimulatory Factor (USF) and TATA motiff are in **bold**. The complete leader sequence is italised with the cap site and splice sites between the individual leaders indicated by double underlining or single underlining respectively.

Fig 3.

Individual sequences of the Promoter cassette components:

I. The 5' (upstream) sequence included in the long cassette.

```
  1   GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAGTCG GAGATGAAGG CACGCGCCCA
 61   GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121   CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181   CCAGTGGTAG
```

II. Sequence including the USF, TATA motiff and sequence to the cap site.

```
  1   CCACGTGACC GGCTTGCGGG TCGGGGGGTA TAAAAGGCGC GGGCCGGGGT GCGTGGCCGT
 61   C
```

III. First leader sequence.

```
  1   AGTTGCTTCG CAGGCCTCGT CACCGGAGTC CGCGTCTCCG GCGTCTCGCG CTGCGGCTGC
 61   ATCTGTGGTC CCGGAGTCTT CAG
```

IV. Second leader sequence.

```
  1   GTCCTTGTTG AGGAGGTACT CCTGATCGCT GTCCCAGTAC TTGGCGTGTG GGAAGCCGTC
 61   CTGATCG
```

V. Third leader sequence.

```
  1   CGATCCTCCT GCTGTTGCAG CGCTTCGGCA AACACGCGCA CCTGCTCTTC GGACCCGGCG
 61   AAGCGTTCGA CGAAGGCGTC TAGCCAGCAA CAGTCGCAAG
```

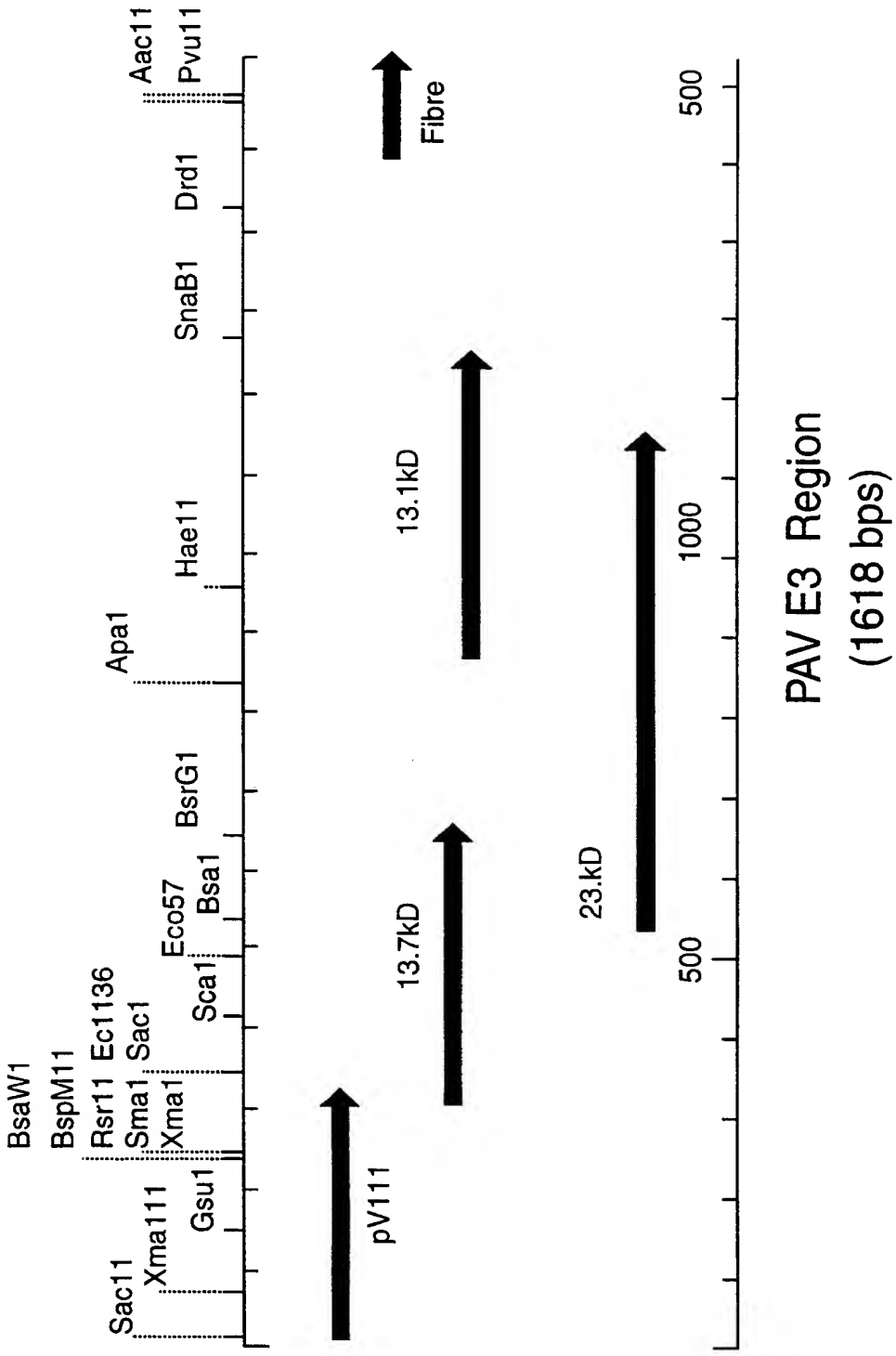
Fig 4.

Sequence of the right hand end of the PAV genome this area being a proposed site for insertion of expression cassettes.

Nucleotide base count 183 A 255 C 306 G 204 T Total 948 bases

```
   1 CATCATCAAT AATATACCGC ACACTTTTAT TGCCCCTTTT GTGGCGTGGT GATTGGCGGA
  61 GAGGGTTGGG GGCGGCGGGC GGTGATTGGT GGAGAGGGGT GTGACGTAGC GTGGGAACGT
 121 GACGTCGCGT GGGAAAATAA CGTGGCGTGG GAACGGTCAA AGTCCGAGGG GCGGGGTCAA
 181 AGTCCGCAGT CGCGGGGCGG AGCCGGCTGG CGGGAATTCC CGGGACTTTC TGGGCGGGTA
                                      EcoRI     SmaI
 241 ATCGTTAACG CGGAGGCGGG GGAATTCCGA TCGGACGATG TGGTACTGAT TAACCGACCG
       HpaI                 EcoRI
 301 CAGGCGTGTC CACATCCGCT GTGGGTATAT CACCGGCGCT CGCGGTGTTC GCTCACACTC
 361 GTCTCGGCGC TGTCACAGAG AGAGACACTG AGAGCGAGAC GAGGAGAAAC CGAAAGCGGG
 421 GCAGGAGGAG TCACCGGGCC ATCTTCCCAT CAGAGCCCTC TCATGGCCCA CGACCGACTG
 481 CTGCTGGCCG CGGTGGCTGA CTGTTGCTCG CCGTGCTCTA TCTGTACTTC GCCTACCTCG
 541 CGTGGCAGGA TCGGGACACT CTTCACACTC AGGAGGCCGC CTCTCCTCGC TTCTTCATCG
 601 GGTCCAACCA CCAGCCCTGG TGCCCGGATT TTGATTGGCA GGAGCAGGAC GAGCACACTC
 661 ACTAGACGTT TAGAAAAAAG ACACACATTG GAACTCATAT ATGTCTGCGG GACCGCATCA
 721 GCAGCCCGGT CTGCTGTTGG CTGCGGGTGA GAGGCCTCCG GTAATTCATC AGAACCGCAT
                                      StuI
 781 TCATCTGCGC CACGTCCCGA CATATGGTGC TGACGTCAGA ACAGCCCAGC GTGATCCTTT
                                      SacIII
 841 TAATGTGCTA GTCTACGTGC CCACTGGGTT TGCTGTGTTT GTGCCGACTG AGCGAGATTT
 901 TCAGAGGAGG GATCTGGTCC GTTTCCAGAC CTGCTGCTTC CGGCATCA
```

The Inverted Terminal Repeat (ITR) is shown in bold. Enzyme sites of interest are underlined with the enzyme name below. Putative TATA for E4 region is also shown.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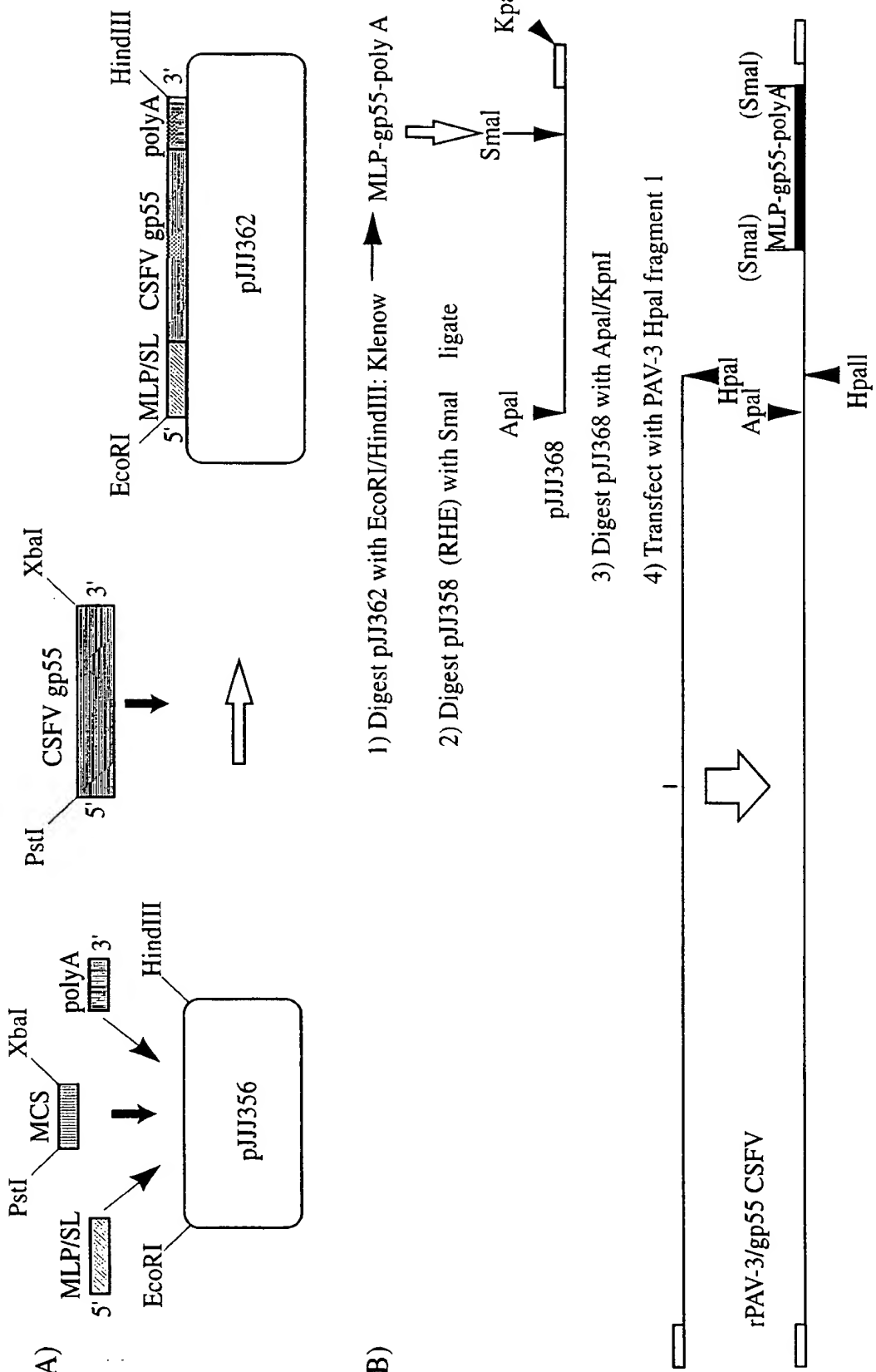
Pvu11
Drd1
SnaB1
Hae11
Apa1
BsrG1
Bsa1
Eco57
Sca1
Sac1
Ec1136
Rsr11
BspM11
BsaW1
Sma1
Xma1
Gsu1
Xma111
Sac11
Fibre
13.1kD
13.7kD
23.kD
pV111
500
1000
500
PAV E3 Region
(1618 bps)

Fig 5.

Fig 6.


A)
MLP/SL
5'
EcoRI
PstI
MCS
XbaI
polyA
3'
HindIII
pJJJ356
PstI
CSFV gp55
5'
XbaI
3'
EcoRI
MLP/SL
5'
CSFV gp55
polyA
3'
HindIII
pJJJ362
B)
1) Digest pJJ362 with EcoRI/HindIII: Klenow
MLP-gp55-poly A
2) Digest pJJ358 (RHE) with SmaI ligate
SmaI
KpaI
ApaI
pJJJ368
3) Digest pJJ368 with ApaI/KpnI
4) Transfect with PAV-3 HpaI fragment 1
HpaI
ApaI
(SmaI)
MLP-gp55-polyA
(SmaI)
HpaII
rPAV-3/gp55 CSFV

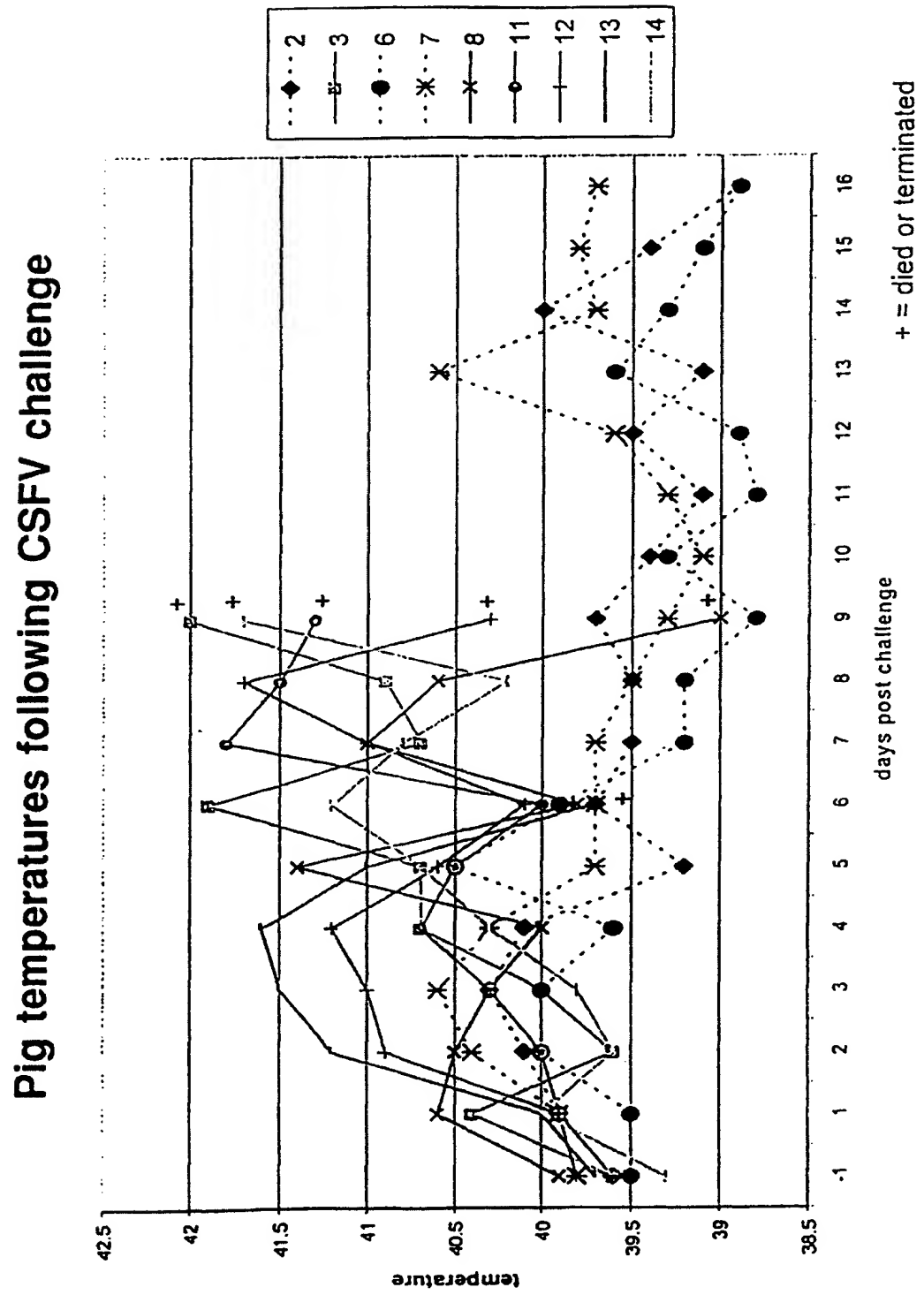
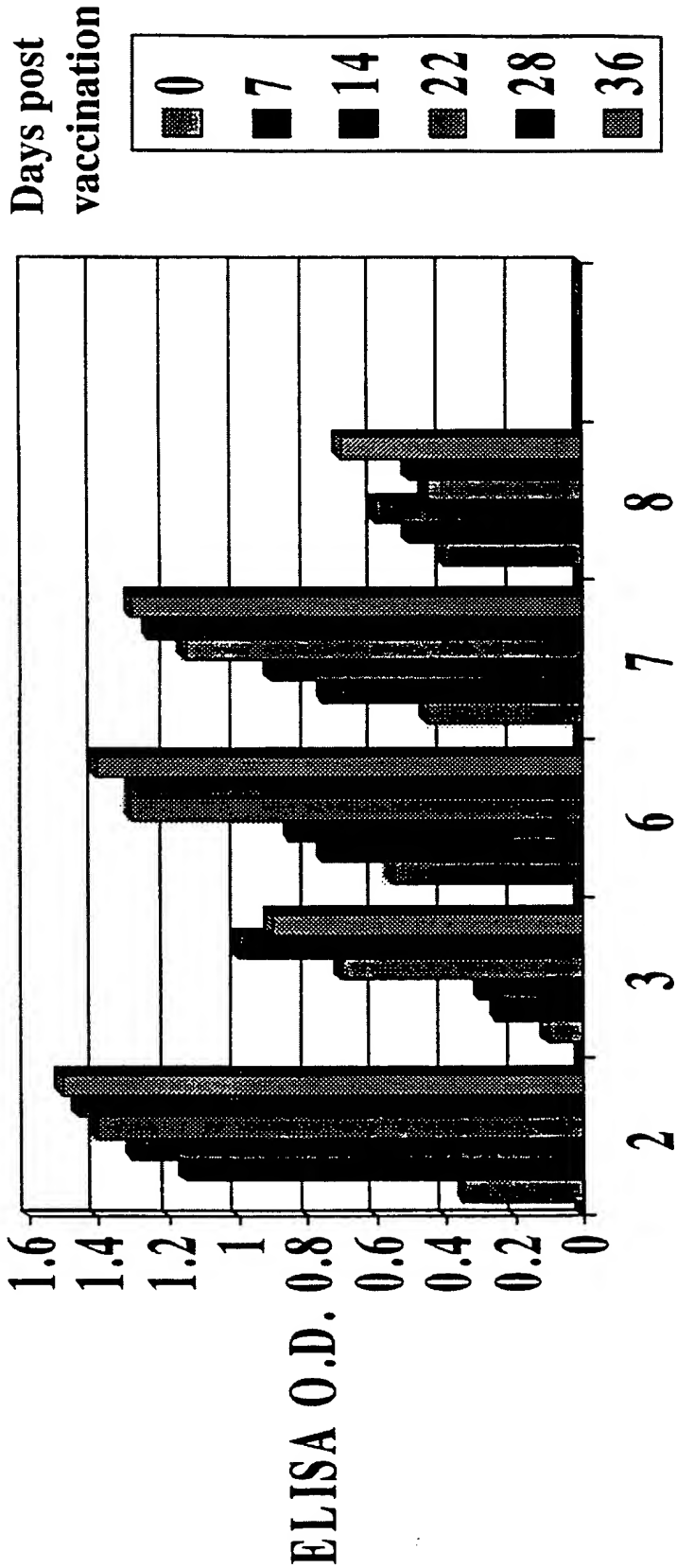

Pig temperatures following CSFV challenge
2
3
6
7
8
11
12
13
14
temperature
42.5
42
41.5
41
40.5
40
39.5
39
38.5
-1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
days post challenge
+ = died or terminated

Fig 7.

Fig 8.

Anti PAV ELISA titres in pigs following rPAV-gp55 vaccination



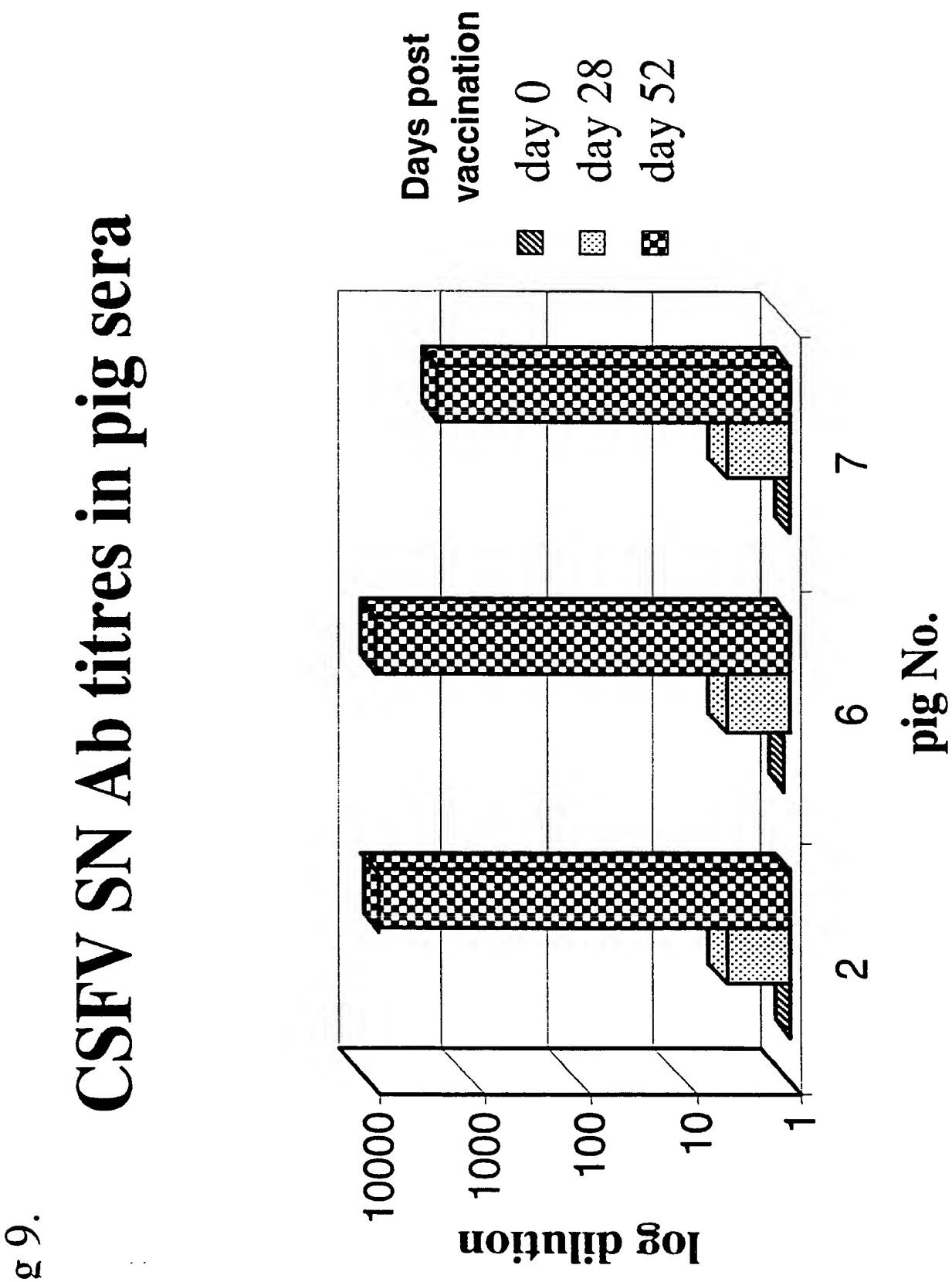

CSFV SN Ab titres in pig sera
Days post vaccination
day 0
day 28
day 52
log dilution
10000
1000
100
10
1
2
6
7
pig No.

Fig 9.

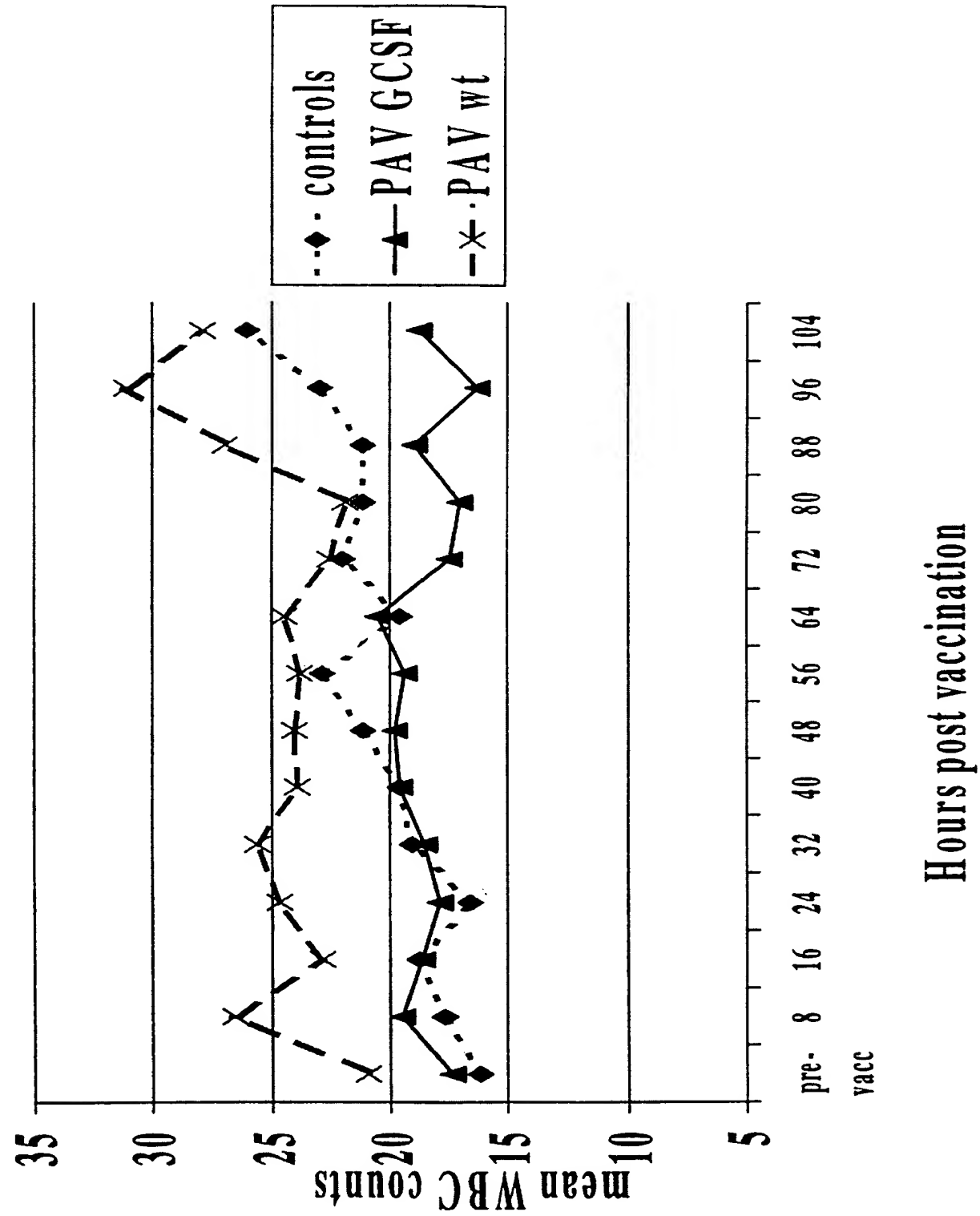

controls
PAV GCSF
PAV wt
mean WBC counts
35
30
25
20
15
10
5
pre-vacc
8
16
24
32
40
48
56
64
72
80
88
96
104
Hours post vaccination

Fig 10.

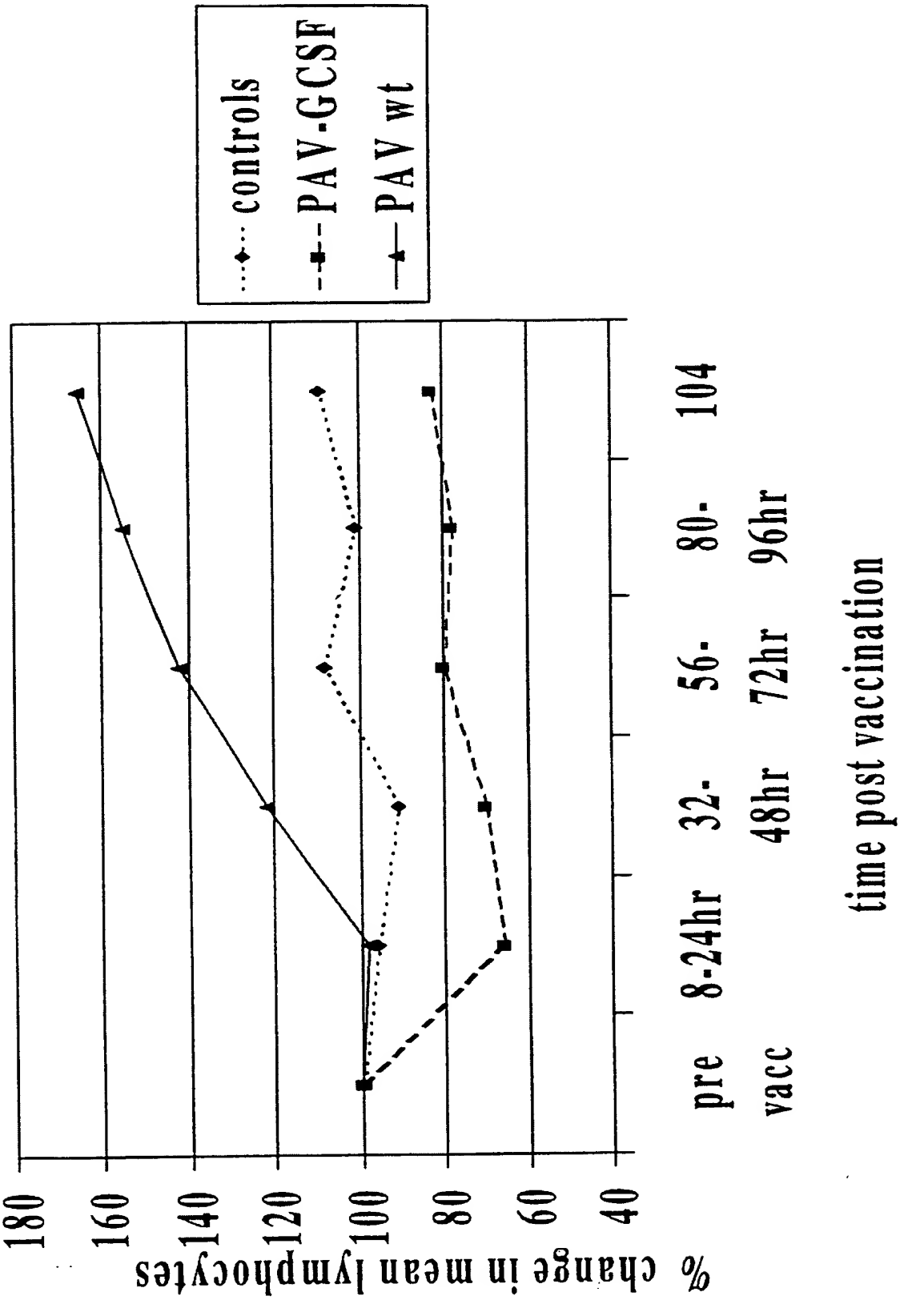

controls
PAV-GCSF
PAV wt
% change in mean lymphocytes
180
160
140
120
100
80
60
40
pre vacc
8-24hr
32-48hr
56-72hr
80-96hr
104
time post vaccination

Fig 11.

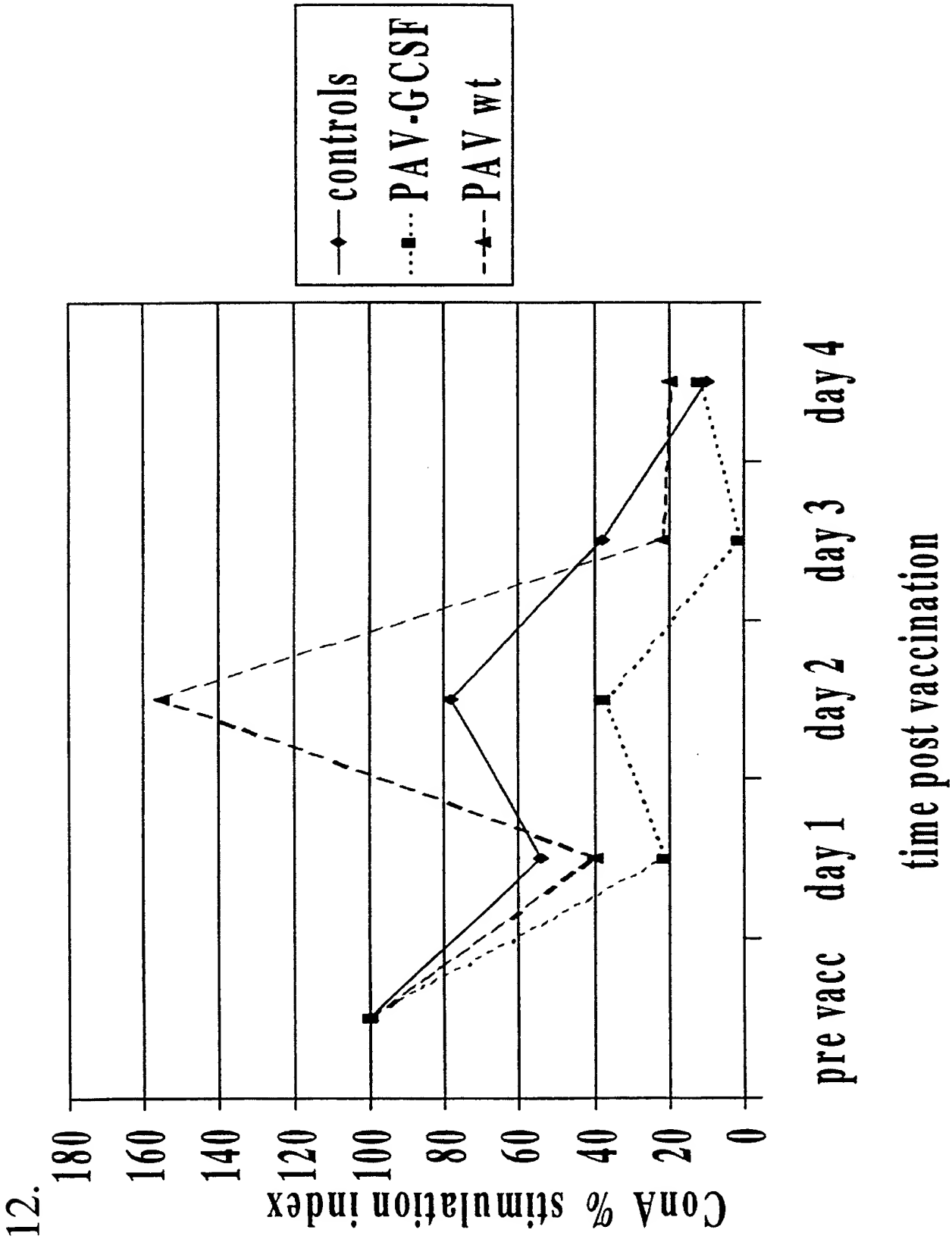

controls
PAV-GCSF
PAV wt
ConA % stimulation index
180
160
140
120
100
80
60
40
20
0
pre vacc
day 1
day 2
day 3
day 4
time post vaccination

Fig 12.

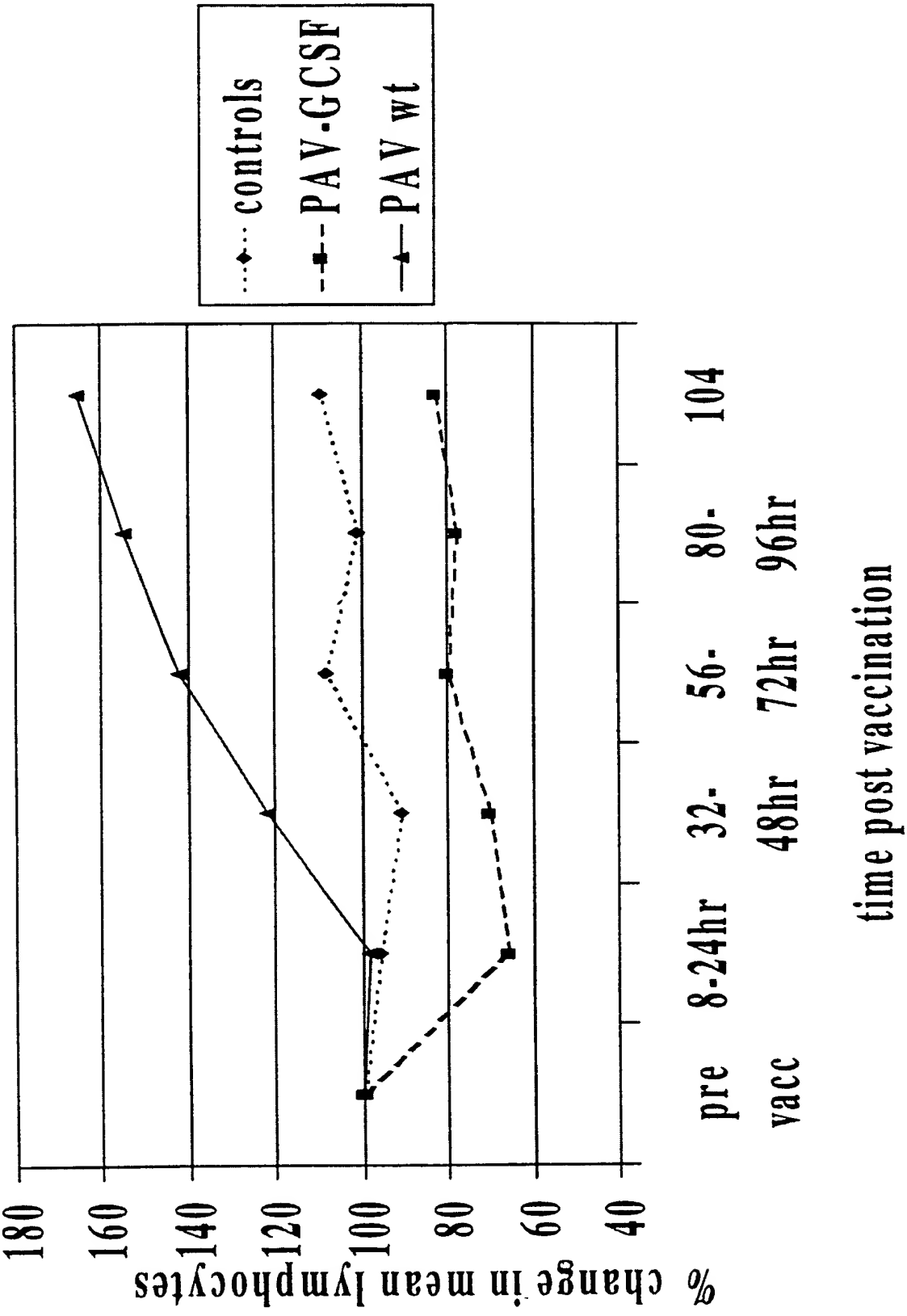

controls
PAV-GCSF
PAV wt
% change in mean lymphocytes
180
160
140
120
100
80
60
40
pre vacc
8-24hr
32-48hr
56-72hr
80-96hr
104
time post vaccination

Fig 13.

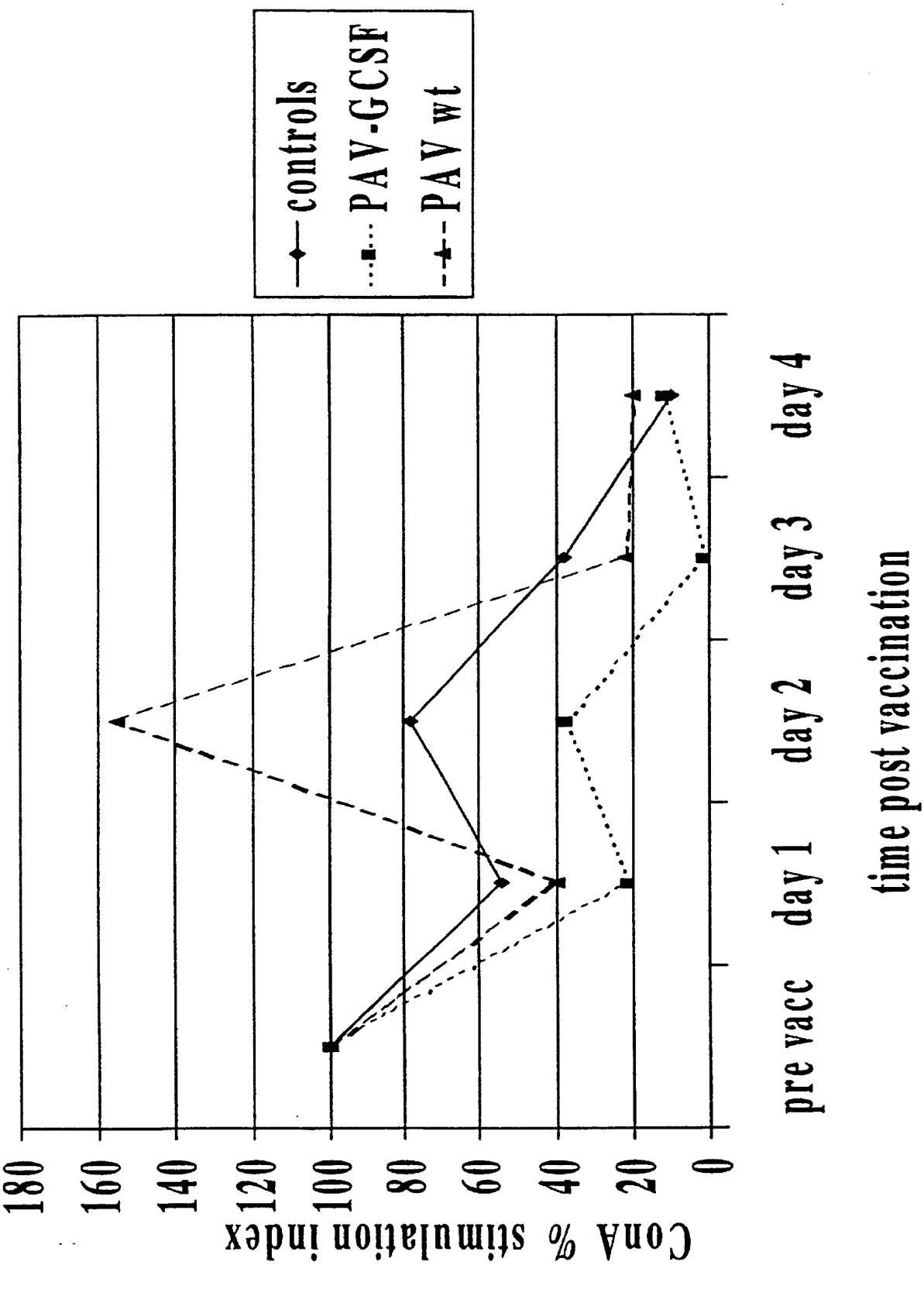

controls
PAV-GCSF
PAV wt
ConA % stimulation index
180
160
140
120
100
80
60
40
20
0
pre vacc
day 1
day 2
day 3
day 4
time post vaccination

Fig 14.